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INVESTIGATIONS ON THE CHEMICAL NATURE OF THE ADRENERGIC RECEPTOR

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "INVESTIGATIONS ON THE CHEMICAL NATURE OF THE ADRENERGIC RECEPTOR", submitted by Manohar R. Parulekar in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

It was the purpose of this research to prepare C^{14}-labelled dibenamine hydrochloride and to explore the conditions necessary for specifically labelling the alpha adrenergic receptors in rabbit aortic tissue with this compound. These studies were intended to lay the groundwork for the eventual isolation of a dibenamine-alpha adrenergic receptor complex.

C^{14}-labelled dibenamine hydrochloride was prepared by a method previously used by Gump and Nikawitz to prepare the unlabelled compound. Benzyl-7-C^{14} chloride (0.25 mc./mM.) was reacted with 2-aminoethanol and the resulting 2-dibenzylaminoethanol treated with thionyl chloride. Recrystallization of the product afforded C^{14}-dibenamine hydrochloride which was found to be identical with dibenamine hydrochloride (SKF) by means of m.p. and infrared studies.

Furchgott demonstrated that when rabbit aortic tissue is exposed to adrenaline (1×10^{-4} g./ml.) for 5 minutes and dibenamine hydrochloride (3×10^{-6} g./ml.) added, the latter drug combines irreversibly with the histamine, serotonin and acetylcholine receptors while the adrenaline receptor

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SKF, Smith Kline and French, Inter-American Corporation.

is partially protected from combination with this drug. We have confirmed these findings and have shown that adrenaline (3.33×10^{-4} g./ml.) is able to partially protect the alpha adrenergic receptor from combination with dibenamine hydrochloride (1×10^{-5} g./ml.).

C^{14}-labelled dibenamine hydrochloride was used to label the alpha adrenergic receptors of the rabbit aortic strip by two procedures. In the first procedure aortic strips were treated with adrenaline (3.33×10^{-4} g./ml.) for 5 minutes and unlabelled dibenamine hydrochloride (1×10^{-5} g./ml.) added. After a 20 minute period strips were washed, allowed to relax, and C^{14}-dibenamine hydrochloride (3×10^{-6} g./ml.) added. As a control the experiments were repeated with the omission of the protecting dose of adrenaline in the first step.

An alternative procedure utilizing C^{14}-dibenamine hydrochloride has been developed which will be of value in the isolation of the alpha adrenergic receptors. In this procedure aortic strips were treated with adrenaline (1×10^{-4} g./ml.) for 5 minutes and C^{14}-dibenamine hydrochloride added. After a 20 minute period the strips were washed, allowed to relax (3 hours) and removed from the bath. As a control the experiments were repeated with the omission of the protecting dose of adrenaline in the first step. Following treatment of experimental and control

strips by this procedure a dibenamine-alpha adrenergic receptor complex should be identifiable, since it should possess the following characteristics: It should be a substance isolated from the control strips with a higher C^{14}-count than a corresponding substance isolated from the experimental strips.

Experimental strips labelled by the first procedure contained significantly greater radioactivity than the control strips, while the converse was the case with strips labelled by the second procedure. The differences in radioactivity in experimental and control strips represent that portion of C^{14}-dibenamine hydrochloride combined with the alpha adrenergic receptor. A detailed consideration of these experiments revealed that the most reliable results are obtained by the second labelling procedure. Consequently the second procedure should be used to label the tissue with C^{14}-dibenamine hydrochloride prior to separating the tissue into its chemical components.

Acknowledgments

The author wishes to express his sincere and deep appreciation to Dr. G. S. Marks for very able guidance both in the course of research and composition of this thesis. He is also very grateful to Dr. D. P. McLeod for help and advice in the electronic equipment used; to Dr. F. H. Henderson, and Dr. E. E. Daniel for critical suggestions; and most of all, to his wife Vijaya for her understanding, unfailing patience and for typing the manuscript.

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I. GENERAL INTRODUCTION

The Mode of Action of Drugs on Cells

It is likely that all the effects of drugs are the consequences of their interaction with molecules of the tissue[1]. Certain drugs such as caffeine and normal aliphatic alcohols only produce effects when given in amounts sufficient to form a monomolecular layer over the entire area of cells. Several hypotheses have been suggested to explain the action of this type of drug[2]. Thus the drug may have an effect on interfacial tension at the cell surface, it may dissolve preferentially in, and affect certain parts of the cell or it might affect the selective passage of various ions through the cell membrane. No obvious relationship between chemical structure and biological activity has been discovered in drugs of this type.

This type of drug action is in contrast to the action of other drugs such as acetylcholine. Although an effective dose of acetylcholine contains a large number of molecules its action is highly selective and confined to a very small area. Thus Clark[3] estimated that a dose of acetylcholine which produces a 50% reduction in the rate of beating of frog's ventricles covers only 0.02% of the surface area of the cells. The idea of a drug producing its effects by an action at a very small part of the cell surface is consistent with the idea that there are active spots, called rece-

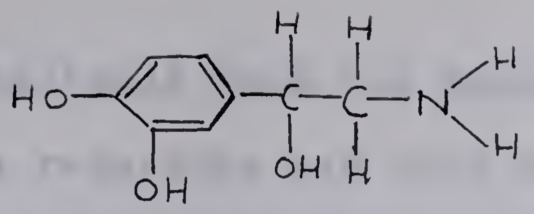
ptors on the cell surface and that the drug acts by forming a complex with these receptors. This idea was suggested by Langley[4] (1905), but is generally associated with the work of Ehrlich who used it as a basis for his work in chemotherapy. It is usual to find that there is a marked variation of biological activity with chemical structure in drugs of this type. This is to be expected because the receptors are assumed to have a definite chemical structure in two or three dimensions and the action of drugs must rest upon their ability to combine with the receptors. Physicochemical properties, however, may be extremely important in determining the rate of transport of a drug to its site of action on or in, the cell.

Adrenotropic Receptors

The adrenotropic receptors are those hypothetical structures located in, on or near the muscle or gland cells affected by the sympathetic transmitter substances. Prior to 1948, the adrenotropic receptors were considered to be of two classes, those whose action resulted in excitation and those whose action produced inhibition of the effector cells. However, Ahlquist's[5] experiments on intact anesthetized animals (Dogs, Cats, Rats & Rabbits) and on isolated tissues (uterus, ureter & gut) indicated that although there are two kinds of adrenotropic receptors

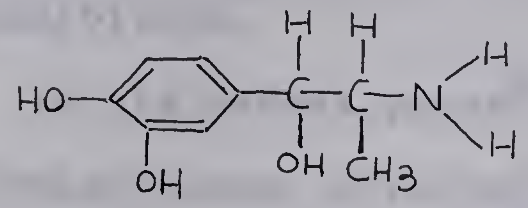
they cannot be simply classified as excitatory or inhibitory since each kind of receptor may have either action depending on where it is found. The evidence for these conclusions was based on the following observations: The potency of a series of structurally related sympathetic amines (1-6; fig. 1) was tested on a variety of blood vessels, myocardium and other smooth muscle preparations. It was concluded that there are two distinct kinds of adrenotropic receptors as determined by their responsiveness to this series of sympathomimetic amines. The alpha adrenotropic receptor is associated with most of the excitatory functions and one important inhibitory function viz., intestinal relaxation. The beta adrenotropic receptor is associated with most of the inhibitory functions and one excitatory function viz., myocardial stimulation. Only the responses mediated through the alpha receptor could be blocked by the then known adrenergic blocking agents which were therefore referred to as alpha receptor blocking agents.

In their original presentation[5] of the alpha and beta adrenergic receptive mechanisms Ahlquist assigned the alpha receptor to the smooth muscle of the intestine but Lands[6] disagreed with this idea. Using the beta receptor blocking agent, dichloroisoproternol (DCI) introduced by Powel and Slater[7], Ahlquist and Levy[8]



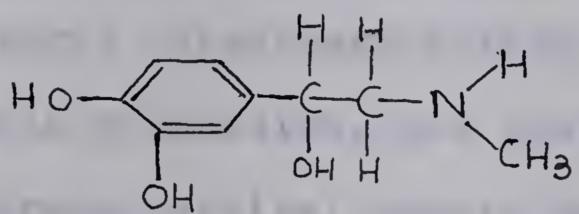
dl-arterenol

(art.)



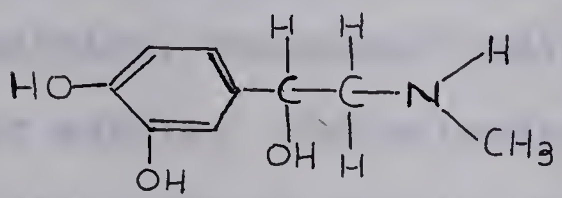
dl-Methyl-arterenol

(methyl-art.)



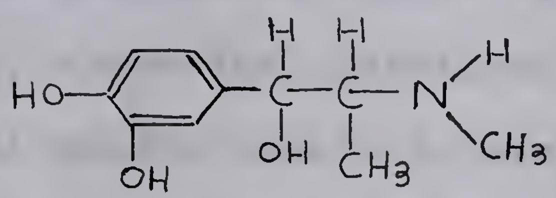
l-epinephrine

(l-epi.)



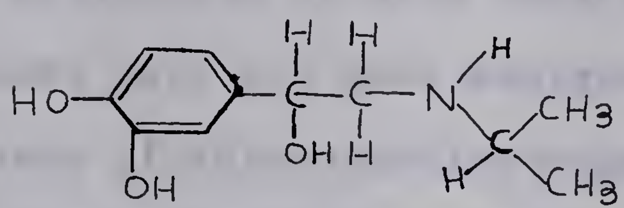
dl-epinephrine

(dl-epi.)



dl-Methyl-epinephrine

(methyl-epi.)



dl-N-Iso-arternol

(N-iso-art.).

<u>Order of Potency</u>

alpha Receptor: l-epi., dl-epi., art., methyl-art., methyl-epi., N-iso-art.

beta Receptor : N-iso-art., l-epi., methyl-epi., dl-epi., methyl-art., art.

Fig. 1

established that the canine ileum has both alpha and beta receptors and both receptors subserve relaxation or inhibition.

In his recent paper[9] Ahlquist has associated the following common effector responses with the alpha receptors: vasoconstriction, mydriasis, intestinal smooth muscle relaxation, and contraction of the nictitating membrane, orbital smooth muscle, splenic smooth muscle, myometrium, retractor penis, seminal vesicle, and pilomotor muscle. The effector responses of vasodilation, bronchial smooth muscle relaxation, myometrial relaxation, myocardial positive inotropic responses and intestinal smooth muscle relaxation have been attributed to the activation of beta receptors. Some adrenergic responses have not been assigned to any specific receptor. Release of adrenocorticotrophic hormone (ACTH) and transient ganglionic blockade have been associated with alpha receptors. Hepatic glycogenolysis and other metabolic effects are regarded as controlled by beta receptors.

Ahlquist[9] mentioned that all uteri contain alpha excitatory and beta inhibitory adrenergic receptors. However, Levy and Tozzi[10] demonstrated, by measuring both isometric and isotonic contractions in isolated rat uterus, that there exist only inhibitory beta receptors irrespective of different phases of the estrus cycle and in

different strains of rat, pseudopregnant or pregnant.

Adrenergic Mechanism at the Alpha Receptor Level

Although intensive studies have been carried out on the kinetics of adrenergic drug interactions with receptors and on the relationship of chemical structure and activity of adrenergic drugs, little insight into the actual nature of alpha and beta receptors has been obtained. Hence Furchgott[11] remarked in 1955, "If our understanding of the actual mechanism of drug action on smooth muscle cells, as well as on other cells to which receptor theory is applied, is to be advanced, working hypotheses on the nature of receptors and receptor-drug reactions will have to be proposed and tested experimentally. This is the direction for the future development of receptor theory." In recent years Belleau[12] proposed a working hypothesis on the nature of the adrenergic receptor which is based on the following considerations: According to Belleau there are two distinct chemical mechanisms of action possible for catecholamines.

(1) The pharmacological response is brought about by a chemical modification of the catecholamine at the receptor site which possesses enzymic activity (fig. 2).

(2) The triggering of the response results only from an electrostatic interaction between the catecholamine and the receptor site (fig. 3).

Fig. 2

Catecholamine + Receptor (with enzymic properties) $\longrightarrow$ Modified Catecholamine + Response

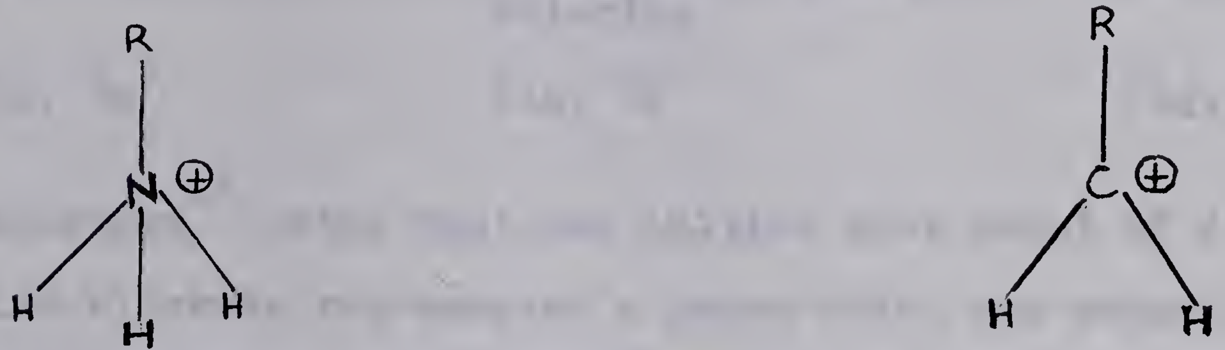
Fig. 3

$$\underset{\text{catecholamine}}{R\text{-}CH_2\text{-}\overset{\oplus}{N}H_3} + \underset{\text{hypothetical site on receptor}}{\overset{\ominus}{O}R} \rightleftharpoons \underset{\text{ion-pair formation between receptor site and catecholamine}}{R\text{-}CH_2\text{-}\overset{\oplus}{N}H_3\overset{\ominus}{O}R}$$

By means of an ingenious series of experiments utilizing deuterium labelled adrenaline, Belleau[13] was able to exclude the possibility that the response was mediated through a chemical change in a catecholamine. Further studies provided strong evidence that the triggering of a response resulted from the electrostatic interaction between catecholamines and anionic sites on the receptor surface. The next experiments of Belleau were directed to demonstrate the presence of an anionic site as a part of the receptor and to elucidate the nature of the site. For this reason he set out to design a suitable antagonist of the catecholamines which would be capable of covalent bond formation with the postulated anionic site through a recognizable chemical reaction. He deduced

that an ideally substituted species isosteric with the ammonium ion was a positively charged carbon atom (fig. 4) since its radius approximates to that of the ammonium ion and it forms a covalent bond with an anionic species.

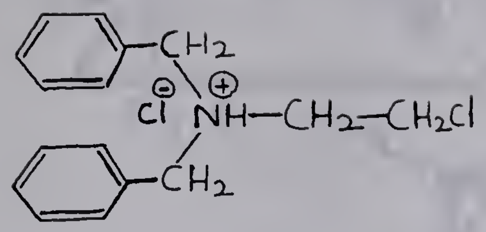
Fig. 4



ammonium ion

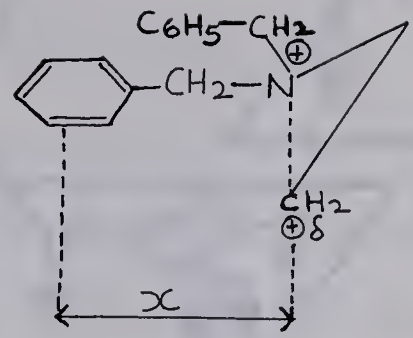
positively charged carbon atom (carbonium ion)

Belleau attempted to design a chemical structure that would generate a reactive carbonium ion under physiological conditions. While trying to do so he realized that such molecules were available under the disguise of the ethyleneiminium ions (fig. 5b) of dibenamine hydrochloride (fig. 5a). Thus dibenamine hydrochloride (fig. 5a), a well known adrenergic blocking agent, corresponded closely to the sought for antagonist. Moreover, it was of great interest that the distance (x) between the carbon atom carrying a partial positive charge and the aromatic nucleus (fig. 5b) corresponded to the distance (x) between the aromatic nucleus and the positively charged nitrogen atom of catecholamines (fig. 5c).



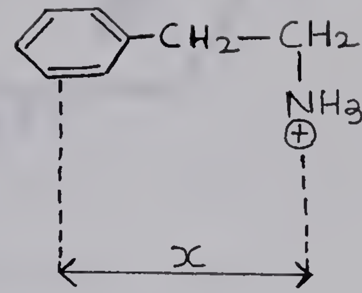
dibenamine hydrochloride

fig. 5a



ethyleneiminium ion of dibenamine hydrochloride

fig. 5b



phenylethylammonium ion

fig. 5c

Nickerson[14] noted that the initial slow onset of dibenamine blockade represented a phase which was competitive with adrenaline while the subsequent prolonged duration of the blockade was of the non-competitive type. Belleau explained this biphasic blocking action of dibenamine as follows: Under physiological conditions dibenamine hydrochloride (fig. 5a) is converted to the ethyleneiminium ion (fig. 5b). In this ion the positive charge of the nitrogen atom is shared with the two carbon atoms of the three membered ring. The ethyleneiminium ion (fig. 5b) first interacts electrostatically with an anionic site of the receptor through its quaternary nitrogen atom as depicted in fig. 6a, and this constitutes Nickerson's first phase of reversible competitive blockade. This is followed by a rearrangement which brings the isosteric carbonium ion adjacent to the anionic site (fig. 6b). It is suggested that alkylation of the anionic site by the carbonium ion ensues and that the period following

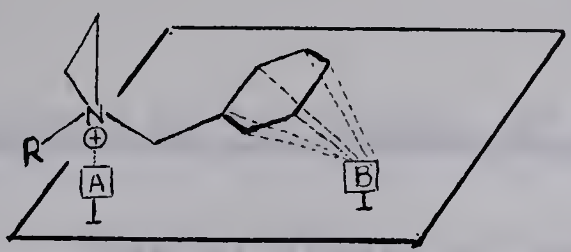


Fig. 6a

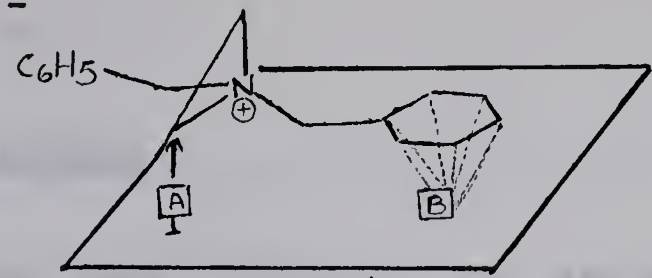


Fig. 6b

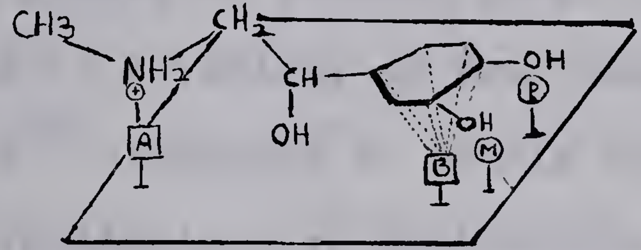


Fig. 6c

alkylation corresponds to the non-competitive phase of blockade described by Nickerson[14]. The interaction of this receptor with adrenaline is envisaged by Belleau as occuring in the manner depicted in fig. 6c.

Nature of the Anionic Site

Belleau considered it likely that the anionic site was either a carboxylate anion or a phosphate anion. On the basis of the protracted action of dibenamine hydrochloride and chemical considerations he deduced that the anionic site was most probably the phosphate anion. Previously Nickerson[14] had considered it likely that dibenamine interacted with the sulfhydryl group of a protein. However, there is no conclusive evidence as to the chemical nature of the receptor substance in a tissue with which dibenamine hydrochloride combines. It was the aim of this thesis to explore the conditions necessary for isolating the alpha adrenergic receptor in order to determine its chemical constitution.

Studies on the Nature of Receptors

Several groups of workers have attempted the study and isolation of a variety of drug receptors. Thus Waser and coworkers[15] attempted to obtain information on the nature and organization of cholinergic receptors in the endplate of rat diaphragm (skeletal muscle) by injecting a minimal lethal dose of C^{14}-calabash curare in the tail veins and subjecting the isolated endplates to radioautography. The borderline between muscle and tendon contained an increased concentration of radiocurare. The concentration of curare in one endplate was measured by a sensitive microdensitometer recording of the film. The number of curare molecules per endplate was calculated as 2.8×10^6 for the minimal lethal dose saturating the endplate. They suggested that the receptor sites for acetylcholine in the muscle endplate are on the outer membrane surface surrounding the exits of "pores". Acetycholine changes the configuration and or charge of the receptor leading to the widening of pores and thus to greater permeability of the membrane to ions. Curare produces a functional block directly at the exits of the pores.

Ehrenpreis and Nachmonsohn[16] isolated a protein (by curare precipitation of the extract from the electric

organ of an electric eel) which they believed to be the cholinergic receptor. However, after studying its binding properties, doubts were expressed by the former author about its biological significance.

Wooley *et al.*[17,18] have evidence that the serotonin receptors from rat's stomach are gangliosides. The receptors are susceptible to destruction by a mixture of neuraminidase and EDTA. By subsequent treatment of the inactivated tissue with purified gangliosides, the sensitivity to serotonin was restored.

The Plan of Work

Our plan for establishing the conditions necessary for the isolation of the alpha adrenergic receptor was based on the following considerations: Furchgott[19] has identified four distinct sets of contraction receptors in the smooth muscle of rabbit aorta which are specific for histamine, acetylcholine, serotonin and adrenergic drugs respectively. It was proposed to prepare C^{14}-labelled dibenamine hydrochloride and to use it to specifically label the alpha adrenergic receptors. It was proposed to treat rabbit aortic strips with adrenaline (3.33×10^{-4} g./ml.) followed by unlabelled dibenamine hydrochloride (1×10^{-5} g./ml.). Under these conditions dibenamine hydrochloride combines with all the

receptors other than the adrenergic receptors. After washing the tissue in order to remove adrenaline and dibenamine the alpha adrenergic receptors would be labelled with C^{14}-labelled dibenamine. An alternative approach to the study of the alpha adrenergic receptor is to treat the aortic tissue with adrenaline (1×10^{-4} g./ml.) followed by C^{14}-labelled dibenamine hydrochloride (3×10^{-6} g./ml.). Under these conditions all the receptors other than the alpha adrenergic receptors would become labelled. This tissue would be compared with the tissue treated directly with C^{14}-labelled dibenamine hydrochloride. The establishment of optimal conditions for labelling the alpha adrenergic receptors would open up the possibility of isolating a dibenamine-alpha-adrenergic-receptor complex.

II. SYNTHESIS OF C^{14}-LABELLED N-(2-CHLOROETHYL) DIBENZYLAMINE HYDROCHLORIDE (DIBENAMINE HYDROCHLORIDE)

The Synthesis of N-(2-Chloroethyl)-Dibenzyl-7-C^{14} Amine Hydrochloride

(A) Outline of method employed and results

C^{14}-labelled N-(2-chloroethyl)-dibenzylamine hydrochloride, dibenamine hydrochloride (IV), has been prepared by a method previously used by Gump and Nikawitz[20] to prepare the unlabelled compound. Benzyl-7-C^{14} chloride (0.25 mc./mM.) (I) was reacted with 2-aminoethanol (II) and the resulting 2-(N,N-dibenzyl)-aminoethanol (III) treated with excess thionyl chloride. Recrystallization of the product from ethanol afforded C^{14}-labelled dibenamine hydrochloride (IV) as white crystals, m.p. 188°-190°. When mixed with a sample of dibenamine hydrochloride (SKF) it had m.p. 188°-190°. The infrared spectrum (fig. 7) of the C^{14}-labelled dibenamine hydrochloride was identical in all respects to that of dibenamine hydrochloride (SKF). It was expected that the specific activity of the dibenamine hydrochloride would be twice that of the benzyl-7-C^{14} chloride since two molecules of this substance are incorporated into one of dibenamine hydrochloride. The specific activity found for this preparation of dibenamine hydrochloride was 0.48 mc./mM. which is in agreement with this expectation within the limits of experimental error.

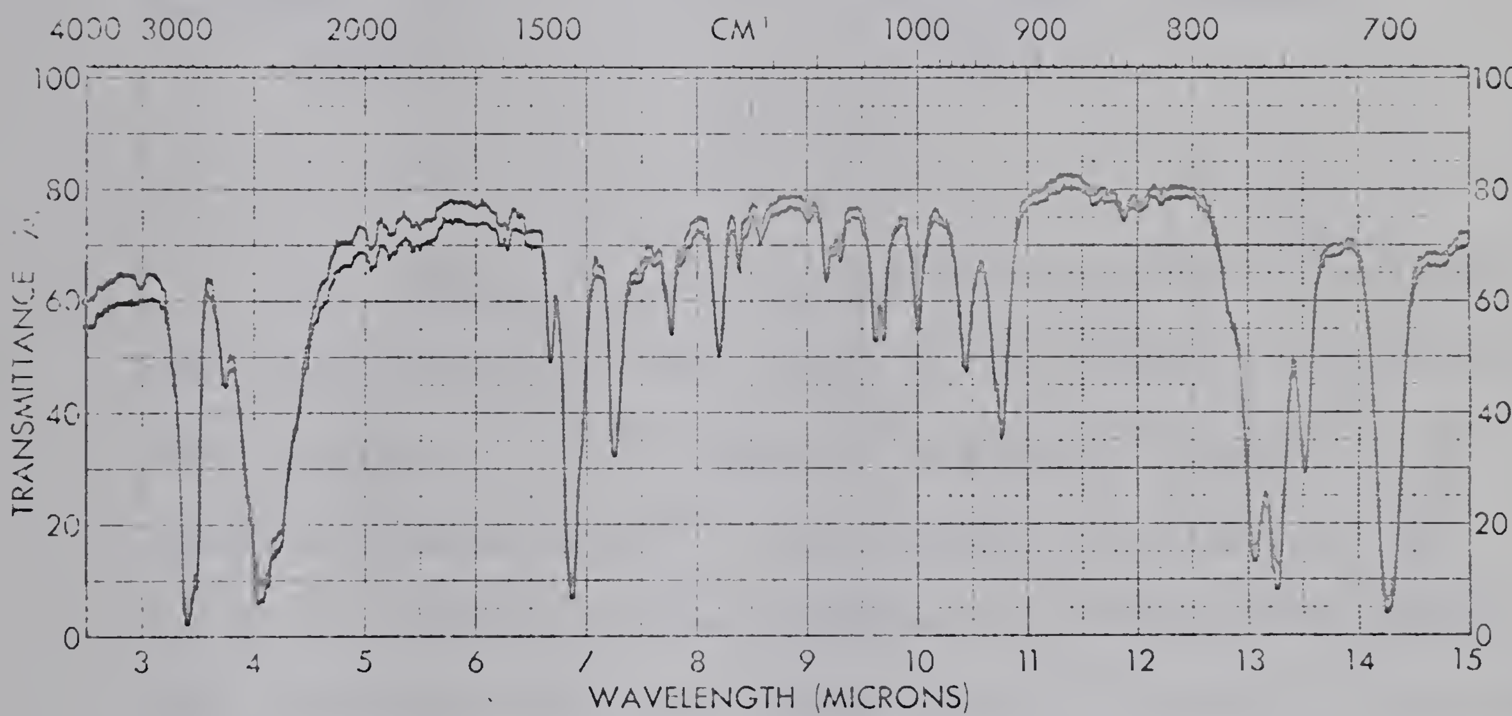
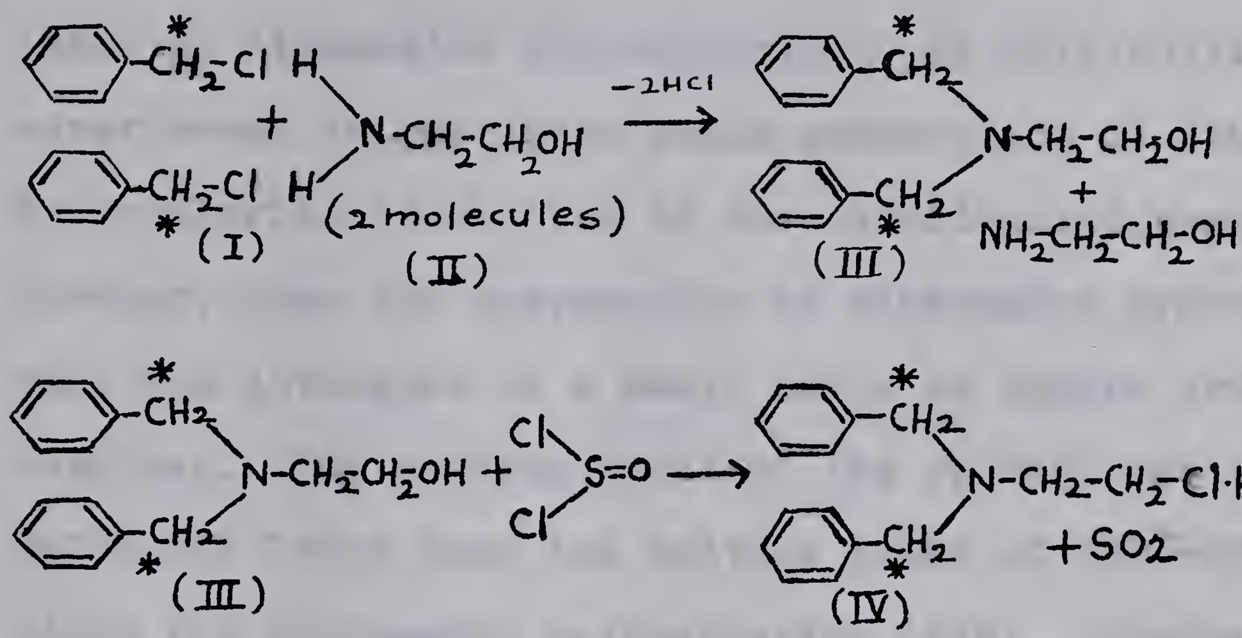


Figure 7. Infrared Spectra in Nujol:

Upper Curve - Dibenamine Hydrochloride (SKF)

Lower Curve - C^{14}-labelled Dibenamine Hydrochloride



The steps involved in the synthesis of C^{14}-dibenamine hydrochloride were based on the general approach to the synthesis of C^{14}-labelled compounds described by Chase and Rabinowitz[21]. Following this approach the first step involved the synthesis of unlabelled dibenamine hydrochloride on a large scale in order to check the method of Gump and Nikawitz and to determine the optimal yields possible. This first step is essential since radioactive syntheses are conducted on such a small scale that the products are often unaccountably lost. By carrying out a preliminary large scale synthesis details can be worked out to eliminate these problems. The second step involved the synthesis on a small scale of unlabelled dibenamine hydrochloride using the actual quantities of materials needed in the final synthesis of radioactive

substance. The final step was the synthesis of C^{14}-labelled dibenamine hydrochloride. No difficulties were experienced in the large scale preparation of dibenamine hydrochloride (described in the experimental section). However, when the preparation of dibenamine hydrochloride was attempted on a small scale an impure product was obtained. The melting point of the product was 178°-180° which was lower than the melting point of 188°-190° determined for dibenamine hydrochloride (SKF). Furthermore the infrared spectrum of this product of low melting point was not identical with that of dibenamine hydrochloride (SKF). A comparison of the infrared spectra was carried out in nujol (fig. 8) and in potassium chloride (fig. 9). In particular the impure product had an absorption band at 3325 cm.$^{-1}$ which was not present in dibenamine hydrochloride (SKF). This is particularly obvious when the spectra of the compounds are compared in nujol (fig. 8). Since absorption in this spectral region is characteristic of the hydroxyl group[22] a possible explanation for our results was the presence of small amounts of the hydrochloride of the intermediate, 2-(N,N-dibenzyl)-aminoethanol as a contaminant in our product. To check this possibility the hydrochloride of 2-(N,N-dibenzyl)-aminoethanol was prepared and its infrared spectrum in nujol recorded (fig. 10). The presence of a prominant band due to hydroxyl absorption at

Figure 8. Infrared Spectra in Nujol:

Upper One - Impure Dibenamine Hydrochloride (m.p. 178°-180°)

Lower One - Dibenamine Hydrochloride (SKF)

Fig. 8

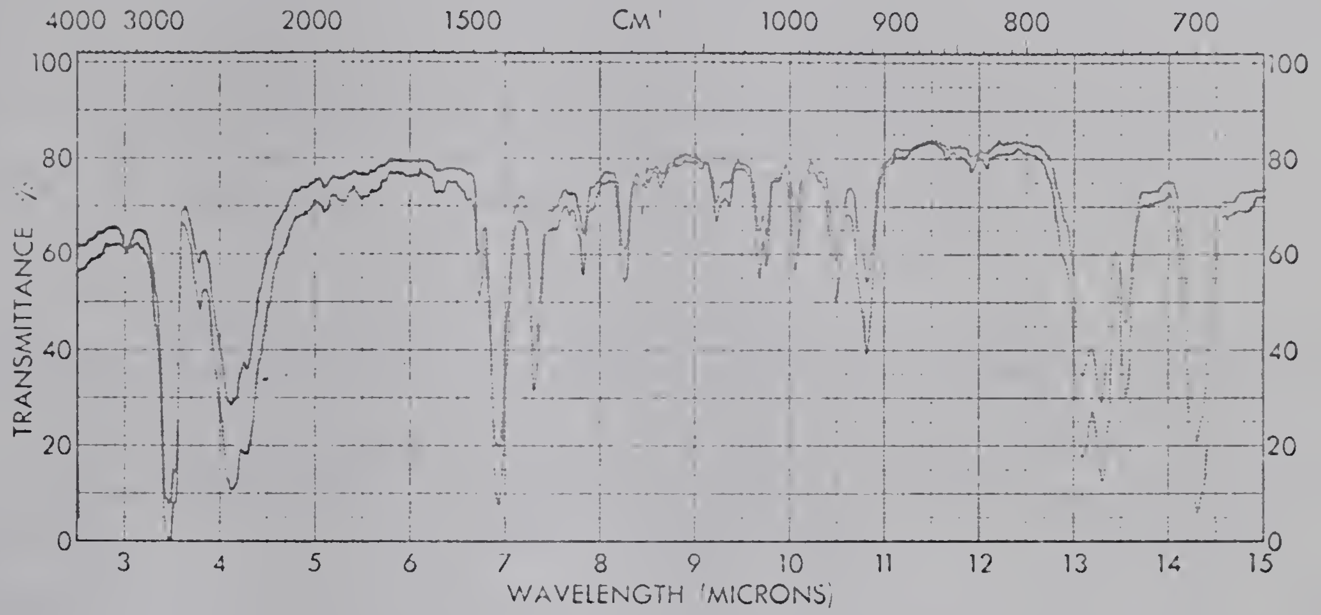


Fig. 9

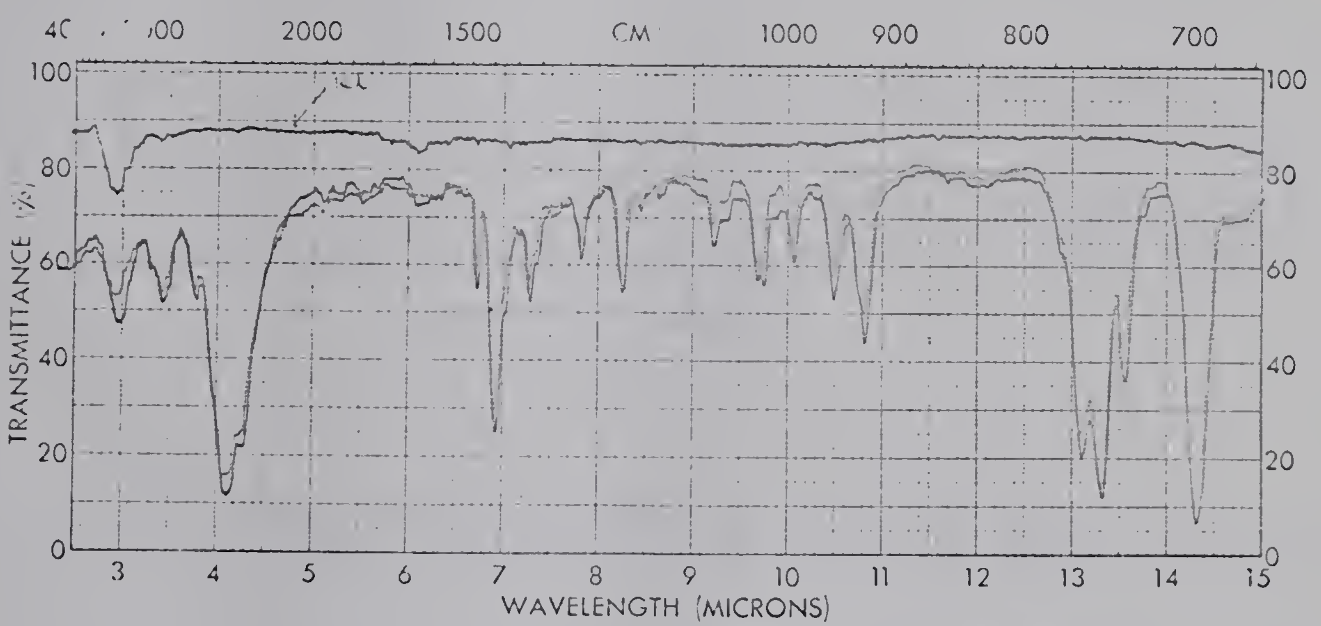


Figure 9. Infrared Spectra in Potassium Chloride

Upper Curve - Potassium Chloride

Middle Curve - Dibenamine Hydrochloride (SKF)

Lower Curve - Contaminated Product of Small Scale Preparation

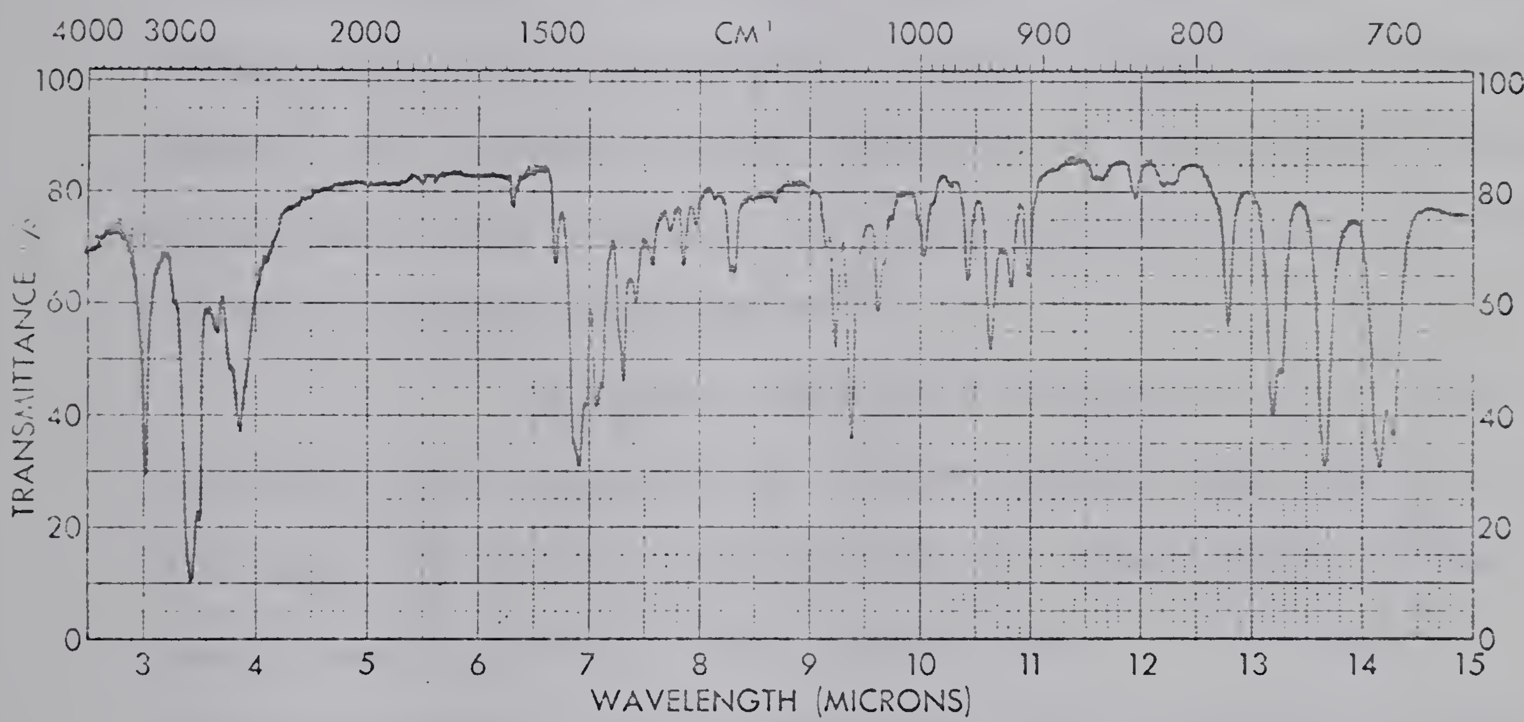


Figure 10. Infrared Spectrum in Nujol of 2-(N,N-Dibenzyl)-Aminoethanol Hydrochloride

3325 cm.$^{-1}$ in the infrared spectrum of this product appeared to confirm this idea. The important question remained as to why this contaminant was present in the small scale preparation despite its absence in the large scale preparation. A consideration of our experimental method revealed that in the small scale preparation, large quantities of chloroform were used as a solvent for 2-(N,N-dibenzyl)-aminoethanol and thionyl chloride in the final step of the synthesis. Since chloroform contains ethanol as a preservative a portion of the thionyl chloride would have been consumed by reaction with ethanol according to the following equation:

$$C_2H_5OH + SOCl_2 \longrightarrow C_2H_5Cl + SO_2 + HCl$$

Therefore the explanation of our results was that the deficiency of thionyl chloride in our final step of the small scale synthesis was responsible for incomplete chlorination of the intermediate 2-(N,N-dibenzyl)-aminoethanol. Accordingly the small scale synthesis was repeated using a considerable excess of thionyl chloride and by this means a pure product was obtained.

(B) EXPERIMENTAL DETAILS OF DIBENAMINE HYDROCHLORIDE SYNTHESIS

(i) Large Scale Preparation

(according to the procedure of Gump and Nikawitz[20])

(a) Synthesis of 2-(N,N-dibenzyl)-aminoethanol. 2-Aminoethanol (66 g., 1 mole) was stirred, heated to about 40° and 128.5 g. (1 mole) of benzyl chloride was added dropwise. At first, the addition of the benzyl chloride was regulated so that the temperature of the mixture rose to about 100°. Later, external heat was applied to keep the temperature at 100°-110° until all of the benzyl chloride had been added. After the mixture had been heated and stirred at 100°-110° for two and one-half hours, a solution of 40 g. of sodium hydroxide in 60 ml. of water was added slowly, and the mixture stirred at 100° for one hour. The oil was extracted from the cold mixture with 100 ml. of benzene, the benzene solution washed with 100 ml. of water, and then dried with anhydrous sodium sulphate. After removal of benzene the crude intermediate was distilled in vacuo to obtain 2-(N,N-dibenzyl)-aminoethanol which boiled at 154° (0.30 mm.), yield 92.6 g. (77%) and infrared spectrum (fig. 11). Gump and Nikawitz record b.p. 190°-195°/5 mm., yield 161 g. (67%).

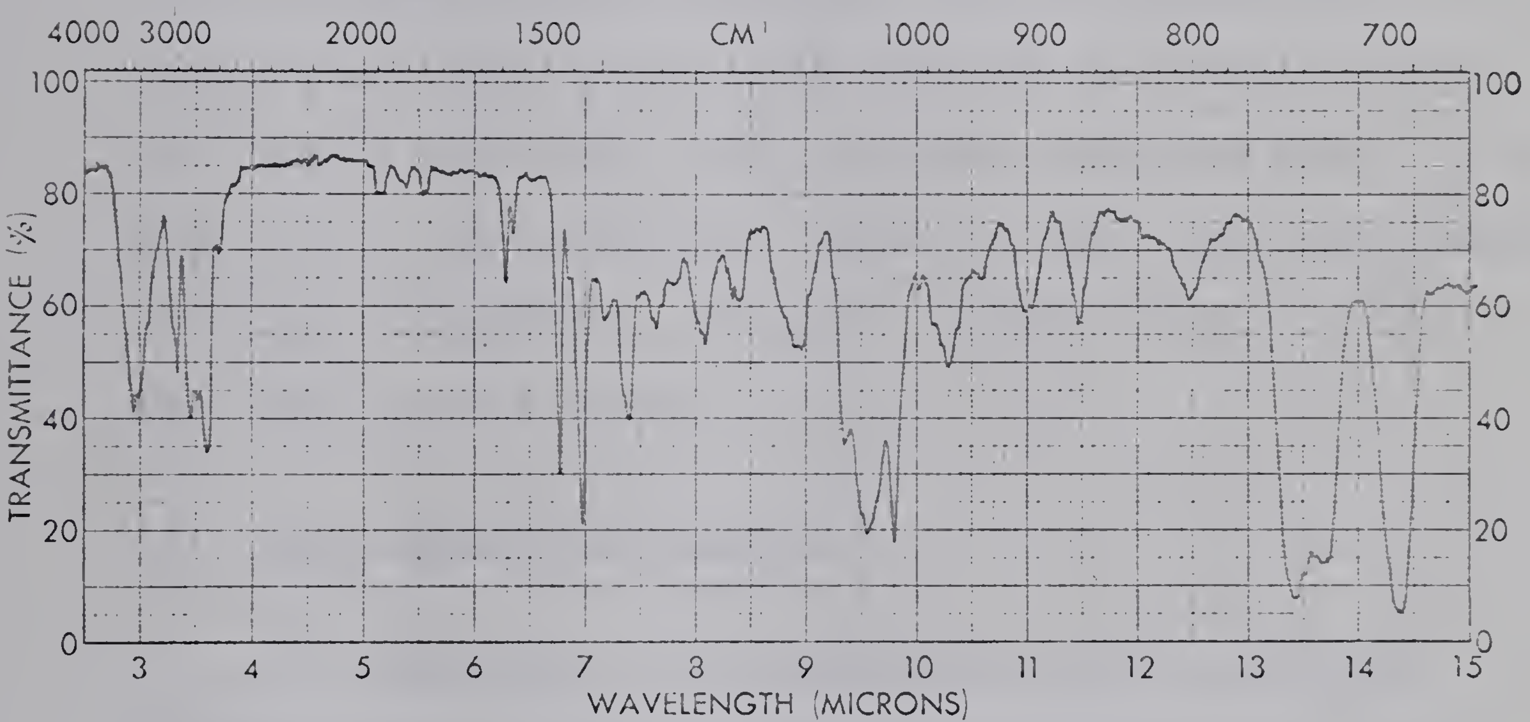


Figure 11. Infrared Spectrum of 2-(N,N-Dibenzyl)-
Aminoethanol (liquid film)

(b) Synthesis of N-(2-chloroethyl)-dibenzylamine (Dibenamine) hydrochloride. A solution of 80 g. (.332 mole) of dibenzylaminoethanol in 84 ml. of chloroform was cooled in an ice-bath, stirred and a solution of 46 g. (.332 mole) thionyl chloride in 50 ml. of chloroform was added during 2 hours. The mixture was stirred for 3 hours at 15°-25°. After 12 hours at room temperature the chloroform was removed under reduced pressure and the residue recrystallized from ethanol and decolorized with the aid of charcaol; m.p. 188°-190°, yield 53%. The infrared spectrum (fig. 7) and m.p. of the product, were identical with those of dibenamine hydrochloride (SKF). Gump and Nikawitz[20] record m.p. 194°-195°; yield 54.7%.

(ii) Small Scale Preparation

(a) Synthesis of 2-(N,N-dibenzyl)-aminoethanol. 2-Aminoethanol (0.24 g.) was heated to about 40° in a semi-micro three-necked flask of 25 ml. capacity and 0.5 g. of benzyl chloride was added dropwise. Later the temperature of the bath was raised and maintained at 100°-110°. After two and a half hours of stirring the mixture at 100°-110°, a solution of 0.156 g. sodium hydroxide in 0.25 ml. aqueous solution was added slowly, and the mixture was stirred at 100° for one hour. The oil was extracted from the cold mixture with a few ml. of benzene, the

benzene solution washed with water in a semi-micro separating funnel, and then dried with anhydrous sodium sulphate in a 10 ml. conical flask. The benzene extract was transferred into bulb # 1 of a micro-distillation apparatus (fig. 13) with a long stemmed Pasteur pipet. Benzene was carefully removed over a hot water bath in vacuo using a water pump. The three bulbed distillation apparatus was then placed in a tin, provided with a thermometer, a mica-window for observation of the distillation and a wire gauze base (fig. 13). Initially the distillation apparatus was adjusted so that bulbs # 1 and # 2 were inside and bulb # 3 outside the tin. The distillation apparatus was evacuated to a pressure of 0.3 mm. and the distillation flask heated with a bunsen burner. At 105^{o}-110^{o} a forerun was collected in bulb # 3, which was cooled with a wet towel. The distillation apparatus was then adjusted so that bulb # 1 remained inside and bulb # 2 and # 3 were outside the tin. Small amounts of the forerun which had condensed in bulb # 2 were driven into bulb # 3 by careful heating of bulb # 2 with a micro-burner. On raising the temperature of the tin further the desired 2-dibenzylaminoethanol distilled as a colorless oily liquid into bulb # 2 at 130^{o}-135^{o}/0.03 mm., yield 80%. Bulb # 2 was isolated from the rest of the distillation apparatus and its contents were poured into a flask. The bulb was rinsed with chloroform to collect small amounts

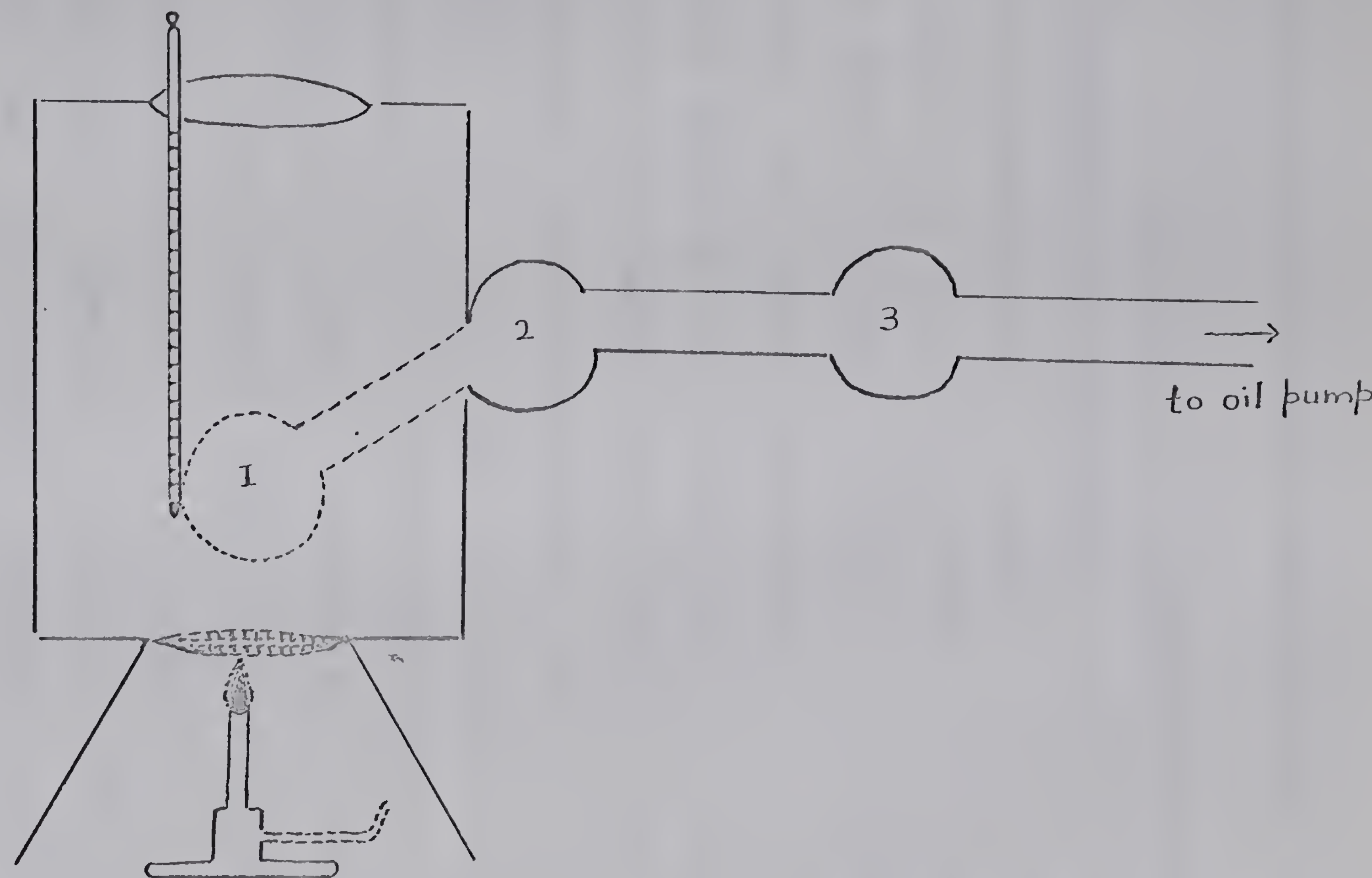


Figure 13. Microdistillation Apparatus Used in Synthesis of Dibenamine Hydrochloride.

of dibenzylaminoethanol which remained in the bulb.

(b) Synthesis of N-(2-chloroethyl)-dibenzylamine (Dibenamine) hydrochloride. A solution of 0.300 g. of dibenzylaminoethanol in about 2 ml. of chloroform was cooled in an ice-bath, stirred and 0.25 ml. of a solution of thionyl chloride (10 ml.) in chloroform (25 ml.) was added. The mixture was stirred for 5 hours at 15°-25°. After keeping for 12 hours at room temperature the chloroform was removed under reduced pressure and the residue recrystallized from alcohol. The product had m.p. of 178°-180°. The low m.p. and the infrared spectrum (fig. 8) indicated that the product was impure. The reasons for obtaining this impure product have been discussed above.

In the revised small scale preparation of dibenamine hydrochloride step (a) was repeated in the manner described above. However, in step (b) 20% excess thionyl chloride was used and a product identical in all respects with dibenamine hydrochloride (SKF) obtained.

(iii) Radioactive Preparation

In addition to the details listed in the small scale preparation of dibenamine hydrochloride, the following precautions were observed: The apparatus was set up in a fume chamber on a special tray lined with thick absorbent

paper on the bottom and aluminium foil on the top. The vial containing 0.164 g.(0.77 mc./mM.) of benzyl-7-C^{14} chloride was chilled in ice and carefully cut open in the fume chamber. 0.168 G. of unlabelled benzyl chloride was added by means of a syringe into the vial. A second syringe with a long needle and flat tip was used to transfer the diluted benzyl-7-C^{14} chloride into the reaction flask containing 0.2 g. of 2-aminoethanol. A second portion of 0.168 g. unlabelled benzyl chloride was then added to the vial with the syringe initially used. The contents of the vial were transferred into the reaction flask by means of the second syringe. The details of the two-stage reaction were the same as in the small scale preparation of unlabelled dibenamine hydrochloride. To prevent possible contamination of the oil or water pumps during the distillation involved in the synthesis a trap, cooled in a mixture of acetone and dry-ice, was interposed between the distillation apparatus and the pump. All the other necessary precautions connected with the handling of C^{14}-labelled compounds[23] were observed. The yield of 2-dibenzyl-7-C^{14} aminoethanol was 85% while that of C^{14}-dibenamine hydrochloride was 49%. The m.p. and the infrared spectrum of this product (fig. 7) were identical with those of dibenamine hydrochloride (SKF).

(iv) Determination of Specific Activity of C^{14}-Dibenamine Hydrochloride

A stock solution of dibenamine hydrochloride (SKF., dried at 100° in vacuo) in ethanol was prepared (1 mg./ml.). A series of solutions of dibenamine hydrochloride in ethanol of different concentrations were prepared from this stock solution and the optical densities at a wavelength of 262 mμ. of these different solutions determined. From this data a calibration curve (fig. 14) was constructed relating concentration of dibenamine hydrochloride to optical density. C^{14}-Dibenamine hydrochloride (2 mg.) was weighed as accurately as possible on a sensitive balance and dissolved in 10 ml. of ethanol. The concentration was checked by observing the optical density and reading the corresponding concentration from the calibration curve. This showed the concentration to be 187.5 μg./ml.. 1 Ml. of the above C^{14}-dibenamine hydrochloride solution (187.5 μg./ml.) was diluted ten times with ethanol and 0.25 ml. of the diluted solution used for radioactivity determination in the Nuclear Chicago Liquid Scintillation Counter. The following steps were involved in the counting procedure:

(a) A standard unquenched C^{14}-sample was used to determine the balance point (at the recommended discriminator setting and at -5°) by adjusting the high voltage of the

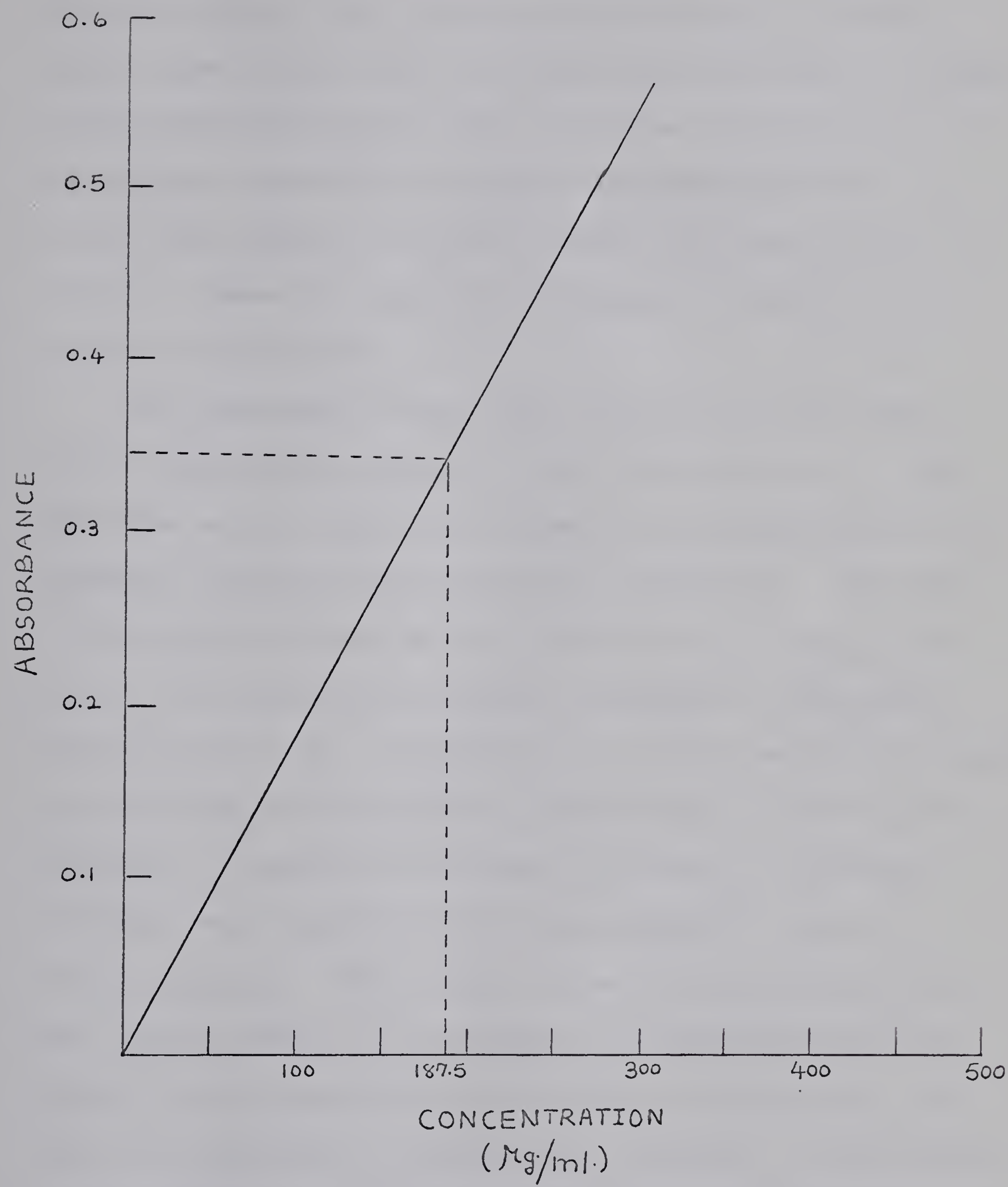


Figure 14. The Absorbance at 262 mµ of Various Concentrations of Dib·HCl.

Data and Gate Photomultiplier tubes to maximize the counting rate. The counting rate is little affected about this balance point by small fluctuations in voltage of the photomultiplier tubes and by a limited degree of quenching. Quenching is caused by substances such as water that absorb excitation energy from the solvent or fluors. Hence the highest efficiency of counting is obtained at this point.

(b) Quenching lowers the counting rate over the entire spectrum of energy of the beta particles. This spectrum can be split up by means of discriminators into suitable regions called windows or channels. The ratio of the counting rate in two channels viz., (L_3-L_4) and (L_3-L_5) of radioactive samples, quenched to different degrees, varies in a way which is a useful measure of degree of quenching or efficiency. Accordingly a series of a standard C^{14}-sample solutions, quenched to different degrees, were counted in the recommended[24] channels (L_3-L_4, L_3-L_5). These count rates are designated C.P.M., and D.P.M. refers to the number of disintegrations per minute in the standard samples prior to quenching. The ratio of the counts in the channel (L_3-L_4) to the counts in the channel (L_3-L_5) for each sample is designated the channel ratio of the sample. A calibration curve was constructed by plotting the percentage efficiencies $\left[\frac{\text{C.P.M.}}{\text{D.P.M.}}\right]$

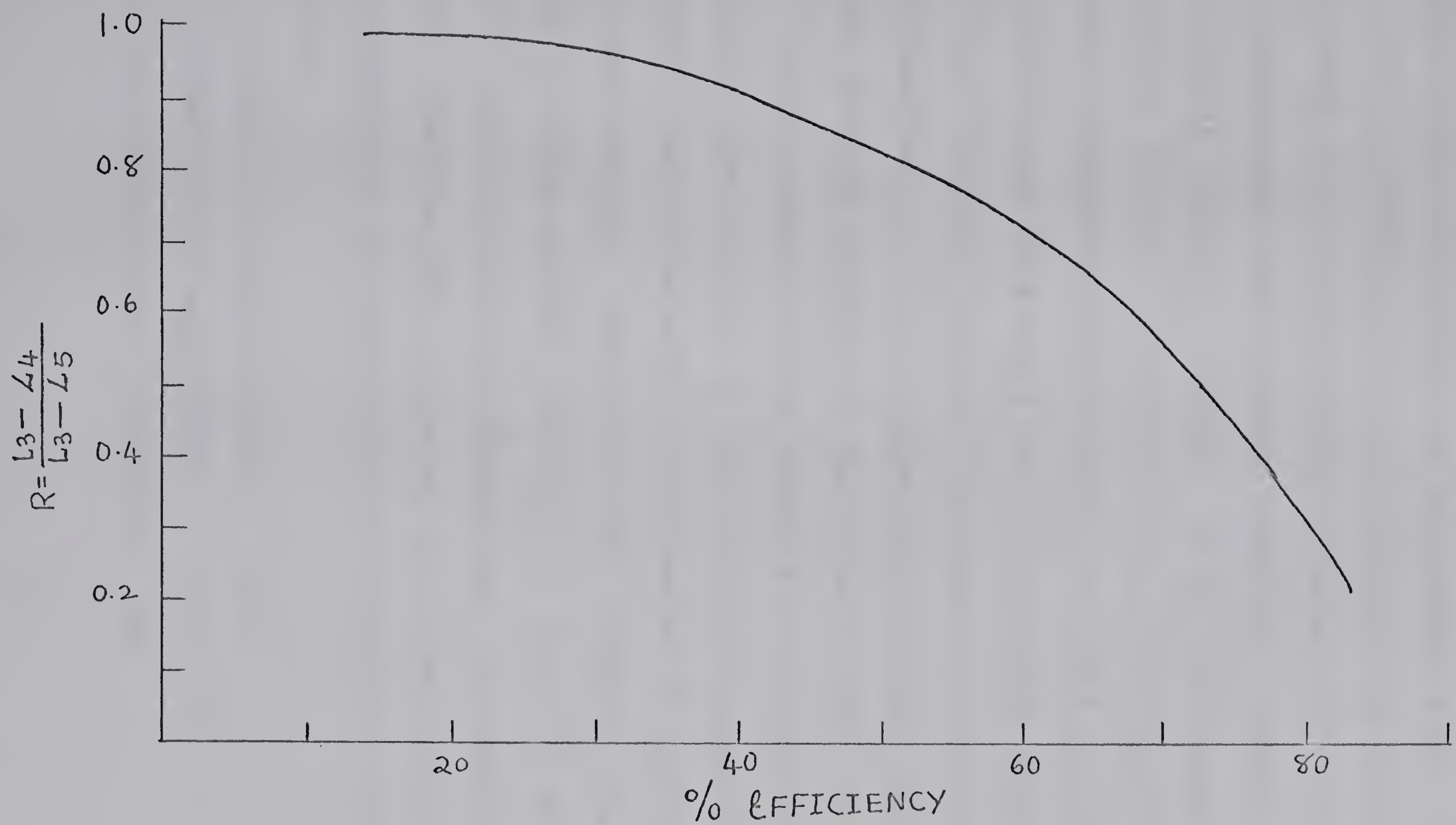


Figure. 15. A Quenching Correction Curve for C^{14} Samples.

of counting of the quenched standard samples against the channel ratios determined for the samples (fig. 15).

(c) A solution of fluors in toluene was prepared by dissolving 6 g. of 2,5-diphenyl oxazole (PPO) and 0.100 g. of 2-p-phenylene bis (5-phenyl oxazole), (POPOP) in reagent grade toluene (1 liter). 30 Ml. of methanol were diluted to 100 ml. with the solution of PPO and POPOP in toluene (designated scintillation solution) and 14.75 ml. of this mixture were added to C^{14}-dibenamine hydrochloride (4.69 g./0.25 ml. of ethanol) for radioactivity measurement. Three samples of C^{14}-dibenamine hydrochloride were counted at the settings outlined in the steps (a) and (b).

(d) The channel ratios observed during the counting procedure were referred to the calibration curve (fig. 15) to obtain the corresponding efficiencies of counting for the three samples. The value of the background was determined by adding ethanol (0.25 ml.) to 14.75 ml. of the scintillation solution and determining the counts in this sample. The observed counts per minute and the correct disintegrations per minute for the samples are recorded in Table 1.

Average D.P.M./μg. dib.HCl. = 3567

Average D.P.M./mg. dib.HCl. = 3567×10^3

Since molecular weight of dib.HCl. = 296

Sample	C.P.M. Channel L_3-L_4	C.P.M. Channel L_3-L_5	Channel Ratio	Per Cent Efficiency	D.P.M.	D.P.M. Corrected for blank	D.P.M./μg of Dib.HCl
A	5319	12,548	0.425	75	16,510	16,472	3516
B	5361	12,868	0.420	76	16,910	16,872	3597
C	5391	12,861	0.420	76	16,872	16,834	3588
Blank	13.80	27.40	0.500	72	38	-	-

Table 1. Determination of Specific Activity of C^{14}-Dibenamine Hydrochloride

$\therefore$ Average D.P.M./mM. dib.HCl. = $296 \times 3567 \times 10^3$

1 mc. = 2.22×10^9 D.P.M.

$\therefore$ The specific activity of the C^{14}-dibenamine hydrochloride

$$= \frac{296 \times 3567 \times 10^3}{2.22 \times 10^9} = 0.48 \text{ mc./mM.}$$

III. STUDY OF THE CONDITIONS NECESSARY FOR SPECIFIC LABELLING OF THE ALPHA ADRENERGIC RECEPTORS

III. Study of the Conditions Necessary for Specific Labelling of the Alpha Adrenergic Receptors

(A) Introduction

A number of isolated preparations have been explored to study the pharmacological receptors for catecholamines and their drug-response curves. Some of the more important preparations are the vas deferens, seminal vesicle, arterial strip, nictitating membrane, sphincter pupillae muscle, perfused rabbit ear vessels and rat hind quarters[25]. The preparation quite sensitive to the action of adrenergic drugs is the rabbit aortic strip popularized by Furchgott[19] for the quantitative study of drugs with vasoconstrictor properties.

The middle coat (tunica media) of the aortic wall consists of layers of cells which have spirally oriented smooth muscle fibers. The smooth muscle cells of the aorta have an autonomic innervation which although not necessary for vascular smooth muscle responses to drugs, is required for high sensitivity[26]. In addition to extreme sensitivity to catecholamines, aortic tissue has a low level of inherent tone. Also once the time has been determined to achieve maximum sensitivity (2 to 3 hours) the responses to drugs remain constant for 4 to 6 hours. Consideration of the above properties favored the adoption of the rabbit aortic strip preparation for labelling of alpha adrenergic receptors.

As mentioned in the introduction Furchgott[19] has identified four distinct sets of contraction receptors in the smooth muscle of rabbit aorta which are specific for histamine, acetylcholine, 5-hydroxytryptamine and adrenergic drugs respectively. Furthermore, he showed that when a rabbit aortic strip was treated with adrenaline (1×10^{-4} g./ml.) for five minutes and the tissue then exposed to dibenamine hydrochloride (3×10^{-6} g./ml.) for 20 minutes, adrenaline protected the alpha adrenergic receptors from combination with dibenamine hydrochloride. However, the histamine, acetylcholine and 5-hydroxytryptamine receptors were not protected by adrenaline and interacted with dibenamine hydrochloride. This suggested a method for specifically labelling the alpha adrenergic receptors with C^{14}-dibenamine hydrochloride. It was proposed to treat the rabbit aortic strips with adrenaline (3.33×10^{-4} g./ml.) for 5 minutes followed by unlabelled dibenamine hydrochloride (1×10^{-5}) for 20 minutes. After washing the aortic strip and allowing it to relax, alpha adrenergic receptors were to be labelled by exposure to C^{14}-dibenamine hydrochloride for 20 minutes.

The first objective of our studies was to determine the degree of protection of the alpha adrenergic receptors afforded by adrenaline against dibenamine hydrochloride and to compare our results with those of Furchgott.

Furchgott[19] defined the degree of protection of the alpha adrenergic receptors as the per cent ratio of sensitivity after exposure of the tissue to the protecting doses of adrenaline and dibenamine hydrochloride to the sensitivity before exposure to these drugs. He arbitrarily defined sensitivity as:

$$\frac{\text{mm. rise of lever during a small contraction}}{\text{concentration of drug used to produce this small contraction.}}$$

He refers to a contraction of less than 10 per cent of the maximal obtained prior to exposure to drugs as a small contraction. During the post-exposure tests the first measurable contractile response is referred to as a small contraction. In our studies it was found more accurate to calculate the degree of protection by a modification of this method. Thus the degree of protection was calculated by means of the following equation: Degree of protection =

$$\left[\frac{\text{Response of tissue to a dose of Ad. after Ad. } (1 \times 10^{-4}) \text{ and Db. } (3 \times 10^{-6})}{\text{Response of tissue to the same dose of Ad. prior to Ad. } (1 \times 10^{-4}) \text{ and Db. } (3 \times 10^{-6})} \times 100\right]$$

where Ad. = adrenaline and Db. = dibenamine hydrochloride.

The degree of protection was determined in the above manner with several small doses of adrenaline and an average value was calculated. Furchgott pointed out that

the degree of protection calculated is the minimum degree of protection. This is due to the fact that the tissue has been desensitized by exposure to a high concentration of adrenaline when the residual sensitivity is determined. Accordingly, the second objective of our studies was to determine the loss of sensitivity of the aortic strip to small doses of adrenaline following exposure to high concentrations of adrenaline.

(B) Experimental

(i) Preparation of Rabbit Aortic Strip[19]

Rabbits weighing between 2 to 3 kg. were killed by rapid decapitation. The descending thoracic aorta was quickly removed and placed in a Petri dish containing Krebs-bicarbonate solution. After removing fat and connective tissue, the uncut portion of the aorta was held gently between the thumb and fingers of the operator's free hand and gradually rotated and moved forward towards the sharp-pointed scissors in such a manner as to permit a continuous spiral incision at a 15^{o} angle relative to the long axis of the intact aorta. A cut strip (30 mm. x 3 mm.) was mounted in the muscle-chamber of 15 ml. capacity containing Krebs-bicarbonate-glucose solution, in the form of a loop as indicated in fig. 16. 95 Per cent O_2 - 5 per cent CO_2 (carbogen) was bubbled through the Krebs-bicarbonate-glucose solution contained in the muscle-chamber and reservoir flask, giving a pH of 7.4. The temperature of the bath was maintained at 37.5^{o} by circulation of warm water in outer jacket of the muscle-chamber from a thermostated water bath (fig. 16). The tissue was loaded with a tension of 2 g. which was maintained constant throughout the experiment.

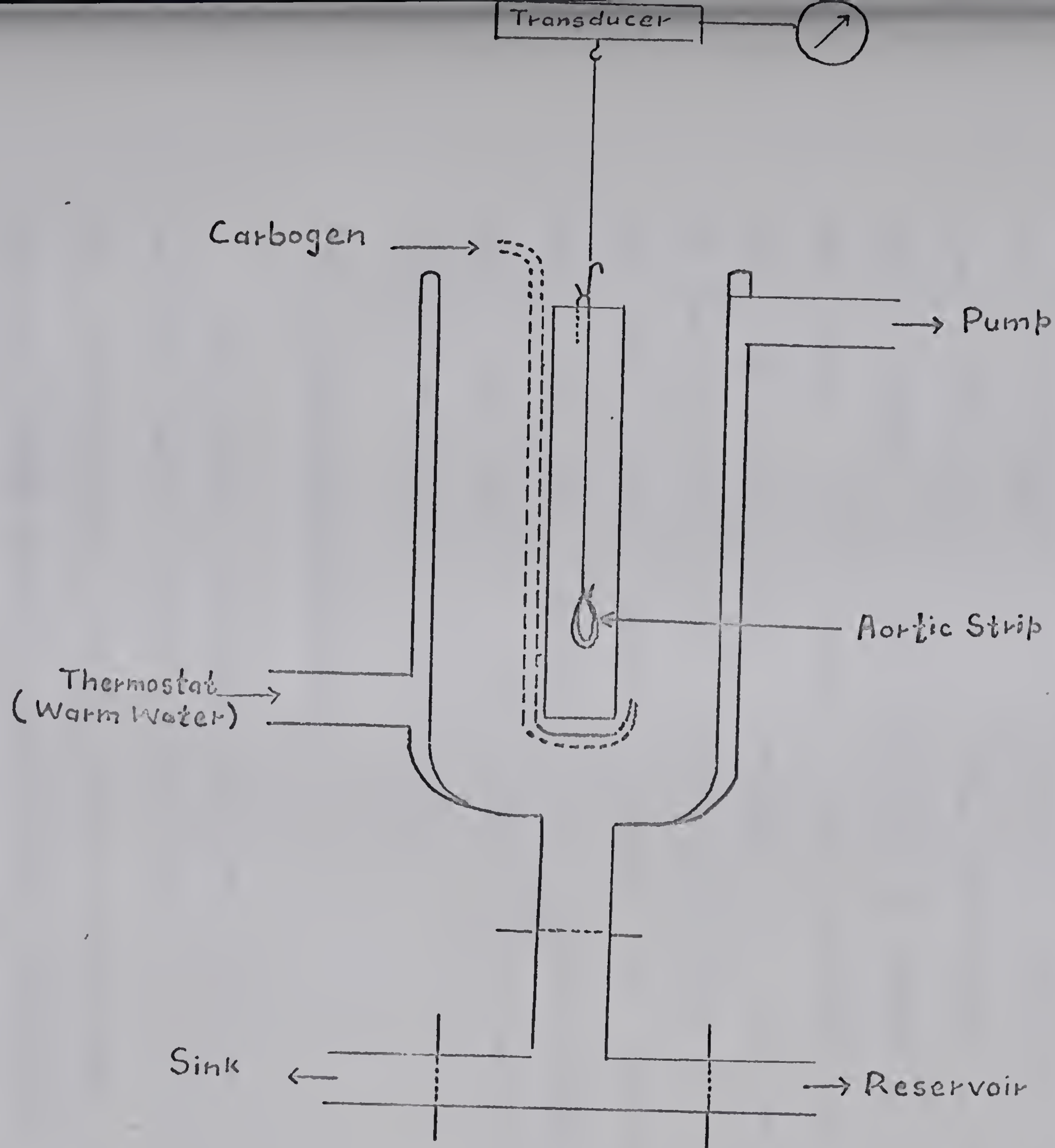


Figure 16. Apparatus Used for Mounting Aortic Strip and Recording Responses to Various Drugs.

(ii) Calibration and Recording

The tissue was attached to a Statham strain gauge transducer by means of a stainless steel wire as shown in fig. 16, and the transducer connected to a Twin-Viso recorder. The recording system was calibrated as follows: The recorder-needle was adjusted to the zero line and a series of weights from 1 to 5 grams added to the transducer. The sensitivity of the instrument was adjusted so that a weight of 1 gram produced an excursion of 1 cm. of the needle. The response of the instrument was linear in the 1 to 5 gram range. A weight of 2 grams was then added to the transducer and a needle deflection of 2 cm. obtained. The needle was adjusted to zero and the instrument again calibrated with a series of 1 to 4 gram weights (1 g.= 1 cm. needle deflection). The recorder speed was 3 cm./min. during measurement of responses.

(iii) Drugs and Solutions

The Krebs-bicarbonate-glucose solution used in the bath had the following composition: 0.116 M NaCl, 0.0046 M KCl, 0.0024 M $CaCl_2.2H_2O$, 0.001 M $MgSO_4.7H_2O$, 0.001 M NaH_2-$PO_4.H_2O$, 0.021 M $NaHCO_3$, and 0.045 M dextrose.

Stock solutions (1 mg./ml.) of l-epinephrine bitartrate, histamine phosphate and 5-hydroxytryptamine creatinine

(ii) Calibration and Recording

The tissue was attached to a Statham strain gauge transducer by means of a stainless steel wire as shown in Fig. 15, and the transducer connected to a Twin-Viso recorder. The recording system was calibrated as follows: The recorder-needle was adjusted to the zero line and a series of weights from 1 to 5 grams added to the transducer. The sensitivity of the instrument was adjusted so that a weight of 1 gram produced an excursion of 1 cm. of the needle. The response of the instrument was linear in the 1 to 5 gram range. A weight of 1 gram was then added to the transducer and a needle deflection of 1 cm. obtained. The needle was adjusted to zero and the instrument again calibrated with a series of 1 to 5 gram weights (1 gm = 1 cm. needle deflection). The recorder speed was 5 mm./sec. during measurement of responses.

(iii) Drugs and Solutions

The Krebs-bicarbonate-glucose solution used in the test had the following composition: 0.118 M NaCl, 0.0047 M KCl, 0.0024 M $CaCl_2.2H_2O$, 0.0012 M $MgSO_4.7H_2O$, 0.0012 M $KH_2PO_4.H_2O$, 0.021 M $NaHCO_3$, and 0.0[illegible] M dextrose.

Stock solutions (1 mg./ml.) of 1-epinephrine bitartrate, histamine [illegible] and 5-hydroxytryptamine creatinine

sulphate (serotonin) were prepared in 0.01 M HCl and kept frozen prior to use. A stock solution of acetylcholine bromide was made in 1% NaH_2PO_4 and stored in the frozen state. A solution of dibenamine hydrochloride was always freshly prepared in 0.01 M HCl. The working solutions of histamine phosphate, acetylcholine bromide and serotonin were made by diluting the stock solutions with isotonic saline. l-Epinephrine bitartrate solution was diluted with saline containing 0.01 M HCl. The dilutions for dibenamine hydrochloride were made with 0.01 M HCl only. All the diluted solutions were kept in an ice-bath for the duration of an experiment. The volume of the muscle bath containing Krebs-bicarbonate-glucose solution was adjusted to 14.8 ml.. The drugs were administered at 15 minute intervals after attainment of maximum sensitivity to a small dose of adrenaline (10^{-8} to 10^{-7} g./ml. of bath fluid). The drugs were made up to a final volume of 0.2 ml. before addition to the bath. Drug concentrations have been expressed as g./ml. of the bath solution and they refer to the concentration of the salt.

(iv) <u>Determination of the Degree of Protection of the Alpha Adrenergic Receptor by Adrenaline (1×10^{-4} g./ml.) Against Dibenamine Hydrochloride (3×10^{-6} g./ml.)</u>

A strip of aortic tissue was mounted in the bath chamber as described above and the tension adjusted to

2 grams. After an average period of approximately 2 hours the tissue had relaxed completely and the tension was readjusted to 2 grams. At this stage the tissue gave consistent responses to small doses of adrenaline. The responses of the tissue to adrenaline, histamine, serotonin and acetylcholine were recorded (fig. 17, a). After exposure of the tissue to adrenaline (1×10^{-4}) for 5 minutes, dibenamine hydrochloride (3×10^{-6}) was added (fig. 17, b). After a 20 minute period the tissue was washed with bath fluid. The washing was repeated at 15 minutes intervals until the tissue relaxed completely. At this point the response of the tissue to adrenaline, serotonin, histamine and acetylcholine were redetermined (fig. 17, c). The tissue was found to be completely insensitive to histamine and serotonin, slightly sensitive to acetylcholine and sensitive to adrenaline. Two similar experiments were carried out and the results are recorded in Table 2, 3, and 4. The average degree of protection found in the first experiment was 43%, in the second experiment 35%, and in the third experiment 32%.

(v) <u>Determination of the Degree of Protection of the Alpha Adrenergic Receptors by Adrenaline (3.33×10^{-4}) Against Dibenamine Hydrochloride (1×10^{-5})</u>

A series of experiments was carried out in an analogous manner to the experiments recorded above but using

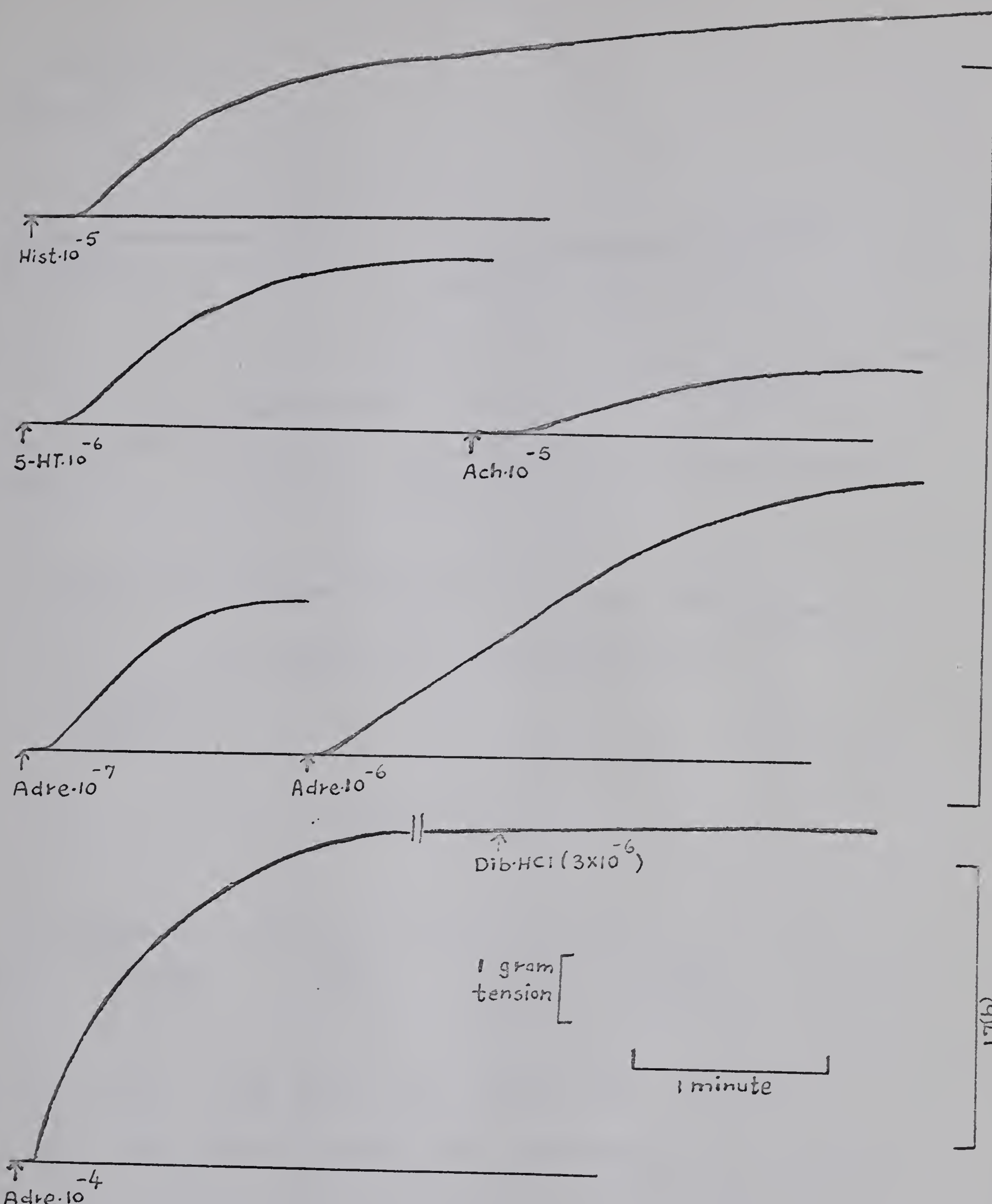


Figure 17· Tracings of Tissue Responses to Hist·, 5-HT·, Ach· and Adre·

(a) before Exposure of Tissue to Adre·(10^{-4}) and Dib·HCl (3×10^{-6})

(b) when Adre·(10^{-4}) and Dib·HCl (3×10^{-6}) Are Added

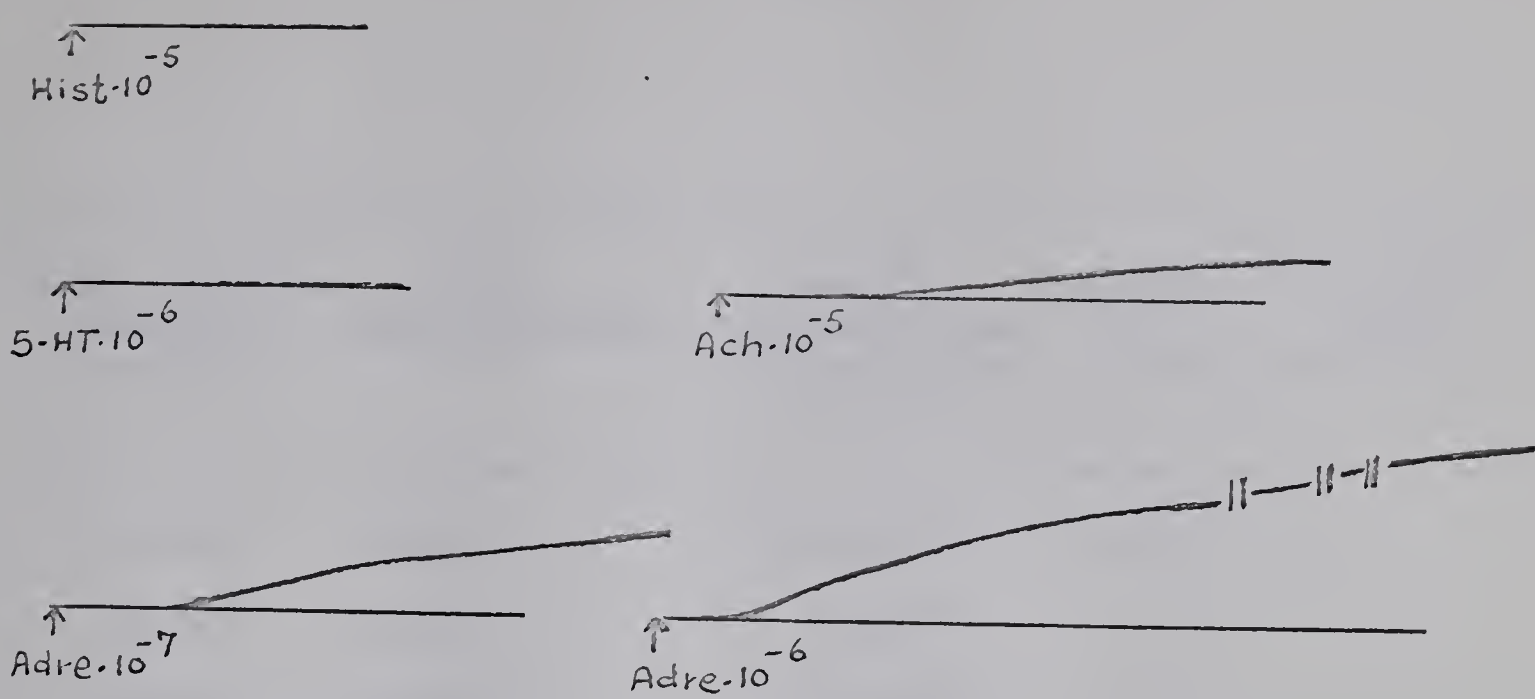
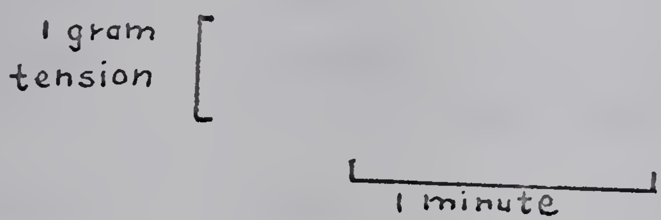
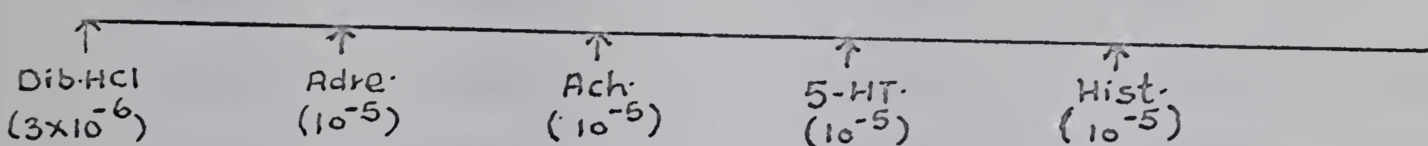


Figure 17(C). Tissue Responses to Drugs after exposure to Adre·(10^{-4}) and Dib·HCl (3×10^{-6})





Tissue Responses to Drugs after Exposure to Dib·HCl alone for 20 Minutes

Time of Drug Adminis- tration	Drug Administered	Dosage Expressed as g/ml.of bath fluid	Tension in grams	Per Cent Pro- tection*
00:00	Ach.	1×10^{-5}	1.70	
00:15	Hist.	1×10^{-5}	2.40	
00:30	5-HT	1×10^{-5}	1.50	
00:45	Adre.	1×10^{-7}	1.00	
01:00	Adre.	1×10^{-6}	2.50	
01:25	Adre.	1×10^{-4}		
01:30	Dib.HCl	3×10^{-6} and washed until	(for 20 minutes the tissue relaxed)	
03:40	Ach.	1×10^{-5}	0.60	
03:50	5-HT	1×10^{-5}	0.0	
04:00	Hist.	1×10^{-5}	0.0	
04:10	Adre.	1×10^{-7}	0.45	45
04:25	Adre.	1×10^{-6}	1.00	40

*As defined in section 3A.

Table 2. Protection of the Alpha Adrenergic Receptors by Adrenaline (1×10^{-4} g/ml.) against Dibenamine Hydrochloride (3×10^{-6} g/ml.).

Time of Drug Adminis- tration	Drug Administered	Dosage Expressed as g/ml. of bath fluid	Tension in grams	Per Cent Pro- tection*
00:00	Hist.	1×10^{-5}	3.70	
00:35	Ach.	1×10^{-5}	0.50	
00:45	5-HT	1×10^{-6}	2.70	
01:15	Adre.	1×10^{-7}	2.00	
01:30	Adre.	1×10^{-6}	4.30	
01:55	Adre.	1×10^{-4}		
02:00	Dib.HCl	3×10^{-6} (for 20 minutes and washed until the tissue relaxed)		
04:00	Ach.	1×10^{-5}	0.20	
04:10	5-HT	1×10^{-6}	0.10	
04:20	Hist.	1×10^{-5}	0.00	
04:30	Adre.	1×10^{-7}	0.60	30
04:45	Adre.	1×10^{-6}	1.7	39.5

*As defined in section 3A.

Table 3. Protection of the Alpha Adrenergic Receptors by Adrenaline (1×10^{-4} g/ml.) against Dibenamine Hydrochloride (3×10^{-6} g/ml.).

Time of Drug Adminis- tration	Drug Administered	Dosage Expressed as g/ml. of bath fluid	Tension in grams	Per Cent Pro- tection*
00:00	Ach.	1×10^{-5}	1.75	
00:10	5-HT	1×10^{-6}	3.10	
00:40	Hist.	1×10^{-6}	2.10	
00:50	Adre.	1×10^{-7}	2.50	
01:10	Adre.	5×10^{-7}	4.10	
01:50	Adre.	1×10^{-4}		
01:55	Dib.HCl	3×10^{-6} (for 20 minutes and washed until the tissue relaxed)		
04:45	Ach.	1×10^{-5}	0.3	
05:50	Hist.	1×10^{-6}	0.0	
05:55	5-HT	1×10^{-6}	0.1	
06:05	Adre.	1×10^{-7}	0.7	28
06:15	Adre.	5×10^{-7}	1.5	36.6

* As defined in section 3A.

Table 4. Protection of the Alpha Adrenergic Receptors by Adrenaline (1×10^{-4} g/ml.) against Dibenamine Hydrochloride (3×10^{-6} g/ml.).

higher concentrations of adrenaline and dibenamine hydrochloride. In these experiments the responses to serotonin, histamine and acetylcholine were not examined. The results of 3 experiments are recorded in Table 5, 6, and 7. The average degree of protection found in the first experiment was 15%, in the second experiment 6%, and in the third experiment 7%.

(vi) Determination of the Loss of Sensitivity of the Aortic Strip to Small Doses of Adrenaline Following Exposure to Higher Doses

The response of the tissue to two low doses of adrenaline was recorded. The tissue was then treated with adrenaline (1×10^{-4}) for 5 minutes, washed, and allowed to relax completely. The response of the tissue to the small doses of adrenaline was then redetermined (Table 8), and found to be approximately 66% of the initial response.

The above experiment was repeated using adrenaline (3.33×10^{-4}) as the high dose of adrenaline. The response of the tissue to the small doses of adrenaline after exposure to the high dose was found to be approximately 19% of the initial response (Table 8).

Time of Drug Adminis-tration	Drug Administered	Dosage Expressed as g/ml. of bath fluid	Tension in grams	Per Cent Pro-tection*
00:00	Adre.	1×10^{-8}	0.70	
00:20	Adre.	1×10^{-7}	1.70	
00:35	Adre.	5×10^{-7}	3.30	
00:50	Adre.	3.33×10^{-4}		
00:55	Dib.HCl	1×10^{-5} (for 20 minutes and washed until the tissue relaxed)		
03:55	Adre.	1×10^{-8}	-	0
04:10	Adre.	1×10^{-7}	0.25	14.7
04:25	Adre.	5×10^{-7}	0.50	15.1

*As defined in section 3A.

Table 5. Protection of the Alpha Adrenergic Receptors by Adrenaline (3.33×10^{-4} g/ml.) against Dibenamine Hydrochloride (1×10^{-5} g/ml.).

Time of Drug Adminis-tration	Drug Administered	Dosage Expressed as g/ml. of bath fluid	Tension in grams	Per Cent Pro-tection*
00:00	Adre.	5×10^{-8}	1.30	
00:10	Adre.	1×10^{-7}	3.30	
00:25	Adre.	3.33×10^{-4}		
00:30	Dib.HCl	1×10^{-5} (for 20 minutes and washed until the tissue relaxed)		
04:50	Adre.	5×10^{-8}	0	0
05:05	Adre.	1×10^{-7}	0.4	12.1

*As defined in section 3A.

Table 6. Protection of the Alpha Adrenergic Receptors by Adrenaline (3.33×10^{-4} g/ml.) against Dibenamine Hydrochloride (1×10^{-5} g/ml.).

Time of Drug Adminis- tration	Drug Administered	Dosage Expressed as g/ml. of bath fluid	Tension in grams	Per Cent Pro- tection*
00:00	Adre.	5×10^{-8}	1.9	
00:20	Adre.	1×10^{-7}	3.00	
00:50	Adre.	3.33×10^{-4}		
00:55	Dib.HCl	1×10^{-5} (for 20 minutes and washed until the tissue relaxed)		
04:00	Adre.	5×10^{-8}	0	0
04:15	Adre.	1×10^{-7}	0.4	13.3

*As defined in section 3A.

Table 7. Protection of the Alpha Adrenergic Receptors by Adrenaline (3.33×10^{-4} g/ml.) against Dibenamine Hydrochloride (1×10^{-5} g/ml.).

Time of Drug Administration	Concentration of Adrenaline in g/ml. of Bath Fluid	Response (Tension in grams)	Final Response to Adrenaline ($5x10^{-7}$) as a % of Initial Response to Adrenaline ($5x10^{-7}$)	Final Response to Adrenaline ($1x10^{-6}$) as a % of Initial Response to Adrenaline ($1x10^{-6}$)
00:00	5×10^{-7}	2.00		
01:00	1×10^{-6}	2.60		
01:30	1×10^{-4}			
03:30	5×10^{-7}	1.40	70	
03:45	1×10^{-6}	1.65		63.5
00:00	5×10^{-7}	1.50		
01:00	1×10^{-6}	1.85		
01:30	3.33×10^{-6}			
03:30	5×10^{-7}	0.20	13.33	
03:45	1×10^{-6}	0.45		24.40

Table 8. Loss of Sensitivity of Aortic Strip to Small Doses of Adrenaline on Exposure to Doses of Adrenaline ($1x10^{-4}$ and $3.33x10^{-4}$ g/ml.) Used in Protection and Labelling Studies.

(C) Discussion of Results

The results of the experiments described in this section have confirmed the report of Furchgott[19] that a concentration of adrenaline of 1 x 10^{-4}g./ml. partially protects the alpha adrenergic receptors from combination with dibenamine hydrochloride (3 x 10^{-6}g./ml.). The histamine and serotonin receptors are completely blocked by dibenamine hydrochloride under these conditions while the acetylcholine receptors are partially blocked. Moreover, the degree of protection obtained in our studies viz. 43, 35 and 32% (average 36%) was in fair agreement with the average value of 34% calculated by Furchgott. In this study the degree of protection afforded by adrenaline (3.33 x 10^{-4}) against dibenamine hydrochloride (1 x 10^{-5}) was found to be 15, 6 and 7% (average 9%) which is considerably lower than that calculated for adrenaline (1 x 10^{-4}) against dibenamine hydrochloride (3 x 10^{-6}). However, it is quite possible that the actual degree of protection obtained under the two sets of conditions is in fact closely similar because of the facts outlined below: Furchgott[19] has pointed out that the mean value for sensitivity remaining in the strips protected by high concentrations of adrenaline gives an underestimate of the actual protection, since exposure to high concentrations of adrenaline alone usually results in 50 to 80 per cent reduction in

sensitivity to low concentrations. Our findings (Table 8) are in agreement with the finding of Furchgott[27] and demonstrate that the response of an aortic tissue to a small dose of adreneline is less after the tissue has been exposed to adrenaline (3.33×10^{-4}) than after exposure to adrenaline (1×10^{-4}). Consequently if the actual degree of protection afforded by adrenaline (3.33×10^{-4}) against dibenamine hydrochloride (1×10^{-5}) was the same as that afforded by adrenaline (1×10^{-4}) against dibenamine hydrochloride (3×10^{-6}) the degree of protection calculated under the first set of conditions would be expected to be considerably smaller than that calculated under the second set of conditions.

IV. LABELLING OF THE ALPHA ADRENERGIC RECEPTORS WITH C^{14}-DIBENAMINE HYDROCHLORIDE

IV. Labelling of the Alpha Adrenergic Receptors with C^{14}-Dibenamine Hydrochloride

(A) Introduction

The next step in our studies involved the labelling of the alpha adrenergic receptors with C^{14}-dibenamine hydrochloride. It was decided to treat the tissue with adrenaline (3.33×10^{-4} g./ml.) and dibenamine hydrochloride (1×10^{-5}) prior to the addition of C^{14}-dibenamine hydrochloride. The reason for choosing these conditions in preference to adrenaline (1×10^{-4}) and dibenamine hydrochloride (3×10^{-6}) was the following: It appeared advantageous to expose the tissue to the higher concentration of unlabelled dibenamine hydrochloride in order to ensure maximal blockade of histamine, serotonin, acetylcholine and non-specific receptor sites prior to addition of labelled C^{14}-dibenamine hydrochloride. The aortic strips treated by this procedure (procedure 1) have been designated as experimental strips. As controls the experiments were repeated with the omission of the protecting dose of adrenaline (3.33×10^{-4} g./ml.) in the initial step. It was anticipated that the experimental strips would contain significantly greater radioactivity than the control strips and for this reason the radioactivity present in the experimental and control strips has been determined.

An alternative method used to study the alpha adrenergic receptor was to treat the tissue with adrenaline followed by C^{14}-dibenamine hydrochloride for a 20 minute period (designated experimental strip) and compare the radioactivity of the tissue with that of a control tissue directly labelled with C^{14}-dibenamine hydrochloride. It was anticipated that the control strip would contain significantly higher radioactivity than the experimental strip and the radioactivity present in the strips has been determined. In the initial studies using this labelling procedure (procedure 2a) the tissue was exposed to adrenaline (1×10^{-4}) followed by C^{14}-dibenamine hydrochloride for a 20 minute period. The tissue was allowed to relax for about 3 hours and the response to lower doses of adrenaline was determined to establish the degree of protection of the tissue. It was possible that a portion of the C^{14}-dibenamine hydrochloride diffused out of the tissue during this period. In order to check this possibility the labelling procedure was modified as follows (procedure 2b): The tissue was exposed to adrenaline (1×10^{-4}) followed by C^{14}-dibenamine hydrochloride for 20 minutes and the tissue was immediately removed for radioactivity determination without determining the degree of protection. The radioactivity of tissues labelled by procedures 2a and 2b was

compared and this enabled an assessment to be made of the amount of C^{14}-dibenamine hydrochloride which diffuses out of the tissue during a 3 hour period.

(B) Experimental

(i) Labelling of the Alpha Adrenergic Receptors with C^{14}-Dibenamine Hydrochloride after Exposure to Adrenaline (3.33×10^{-4}) and Unlabelled Dibenamine Hydrochloride (1×10^{-5})

Procedure 1: A rabbit aortic strip was mounted in a chamber of 15 ml. working volume, attached to a transducer which was connected to a recording system. The details of the procedure have been outlined in section 3B. The response of the strip to several small doses of adrenaline was recorded. The strip was then treated with adrenaline (3.33×10^{-4}) for 5 minutes and unlabelled dibenamine hydrochloride (1×10^{-5}). After a 20 minute period the tissue was washed, allowed to relax completely (approximately 3 hours) and the response to the small doses of adrenaline previously used was recorded. The degree of protection was calculated as described in section 3A. The adrenaline receptor was labelled by the addition of C^{14}-dibenamine hydrochloride (3×10^{-6}). After a 20 minute period the tissue was washed 6 times with the bath fluid, removed from the bath and dried from the frozen state. The tissue treated in the above manner has been designated the experimental strip. As controls the experiments were repeated with the

omission of the protecting dose of adrenaline (3.33×10^{-4}) in the initial step. In any particular experiment the experimental and control strips were from the same rabbit. The degree of protection obtained in five experiments of this type is recorded in Table 9. A further three experiments of the same type carried out by Mr. Jim Wright are included in the Table for comparison purposes.

(ii) Labelling of the Alpha Adrenergic Receptors with C^{14}-Dibenamine Hydrochloride without prior Exposure of Tissue to Unlabelled Dibenamine Hydrochloride

Procedure 2a: The response of the tissue to several small doses of adrenaline was recorded. The strip was then treated with adrenaline (1×10^{-4}) for 5 minutes and C^{14}-dibenamine hydrochloride (3×10^{-6}) added. After a 20 minute period the tissue was washed, allowed to relax completely and the response to small doses of adrenaline previously used was recorded. The tissue was removed from the bath and dried from the frozen state. The tissue treated in this manner has been designated the experimental strip. As a control the experiment was repeated with the omission of adrenaline (1×10^{-4}) in the first step. The degree of protection obtained in 5 experiments of this type is recorded in Table 10.

Average Degree of Protection***	Dry Weight of Aortic Strip		Total D.P.M./mg. of Dry Weight of Strip	
	Control Strip	Experimental Strip	Control Strip	Experimental Strip
11	21.3	22.7	125	122
17	23.0	20.4	151	216
12	14.4	17.5	146	298
16	20.1	25.0	233	208
9	11.7	13.8	176	212
20*	17.3	16.1	106	201
33*	16.7	17.3	182	208
19*	14.2	12.3	124	178
Mean $\pm$S.D.	17.3$\pm$3.6	18.1$\pm$4.1	155$\pm$38	205$\pm$45
	P value**		0.01 <P <0.025	

*These three experiments were done by Mr. Jim Wright.
**P value was obtained by means of a one tailed t-test.
***As defined in section 3A.

Table 9. The Disintegrations per minute Detected in Control and Experimental Rabbit Aortic Strips (Procedure 1).

Average Degree of Protection**	Dry Weight of Aortic Strip		Total D.P.M./mg. of Dry Weight of Strip	
	Control Strip	Experimental Strip	Control Strip	Experimental Strip
38	15.4	10.7	171	137
43	13.2	10.9	156	99
20	16.5	15.9	179	151
41	13.1	12.0	212	158
40	17.0	19.0	185	142
Mean ±S.D.	15.2±1.6	13.7±3.2	181±19	137±21
	P value*		$0.005 < P < 0.01$	

*P value was obtained by means of a one tailed t-test.
**As defined in section 3A.

Table 10. The Disintegrations per minute Detected in Control and Experimental Rabbit Aortic Strips (Procedure 2a).

Procedure 2b: This method differed from the procedure 2a in that after exposure of the strip to C^{14}-dibenamine hydrochloride for 20 minutes, the strip was washed once, removed from the bath and dried from the frozen state. The degree of protection could not be calculated under these conditions.

(iii) Determination of Radioactivity in Experimental and Control Strips

Strips which had been dried from the frozen state were weighed and dissolved at 70° in one ml. of 5N KOH per 50 mg; 0.2 ml. aliquots were added to 17.8 ml. of scintillation solution and the radioactivity determined in a liquid scintillation counter. The scintillation solution was prepared as described in section 2B. An unlabelled aortic strip was treated in the same manner as the labelled strip and used to obtain the background count. The samples were counted for a period of 10 minutes. This time period was calculated to reduce the standard error of the counting to less than 2%. The results obtained using procedure 1 are recorded in Table 9, using procedure 2a in Table 10 and using procedure 2b in Table 11.

	Dry Weight of Aortic Strip		Total D.P.M./mg. of Dry Weight of Strip	
	Control Strip	Experimental Strip	Control Strip	Experimental Strip
	10.25	10.65	297	268
	12.25	15.30	266	227
	10.75	11.25	279	177
	14.00	9.65	205	164
	14.25	14.10	292	221
Mean $\pm$S.D.	12.30$\pm$1.8	12.19$\pm$2.4	268$\pm$37.3	212$\pm$42
P value*			$0.025 < P < 0.05$	

*P value was obtained by means of a one tailed t-test.

Table 11. The Disintegrations per minute Detected in Control and Experimental Rabbit Aortic Strips (Procedure 2b).

(C) Results and Discussion

The radioactivity present in the experimental and control strips labelled according to procedure 1 is recorded in Table 9. A significant increment in radioactivity ($P < 0.025$) was noted in the experimental strips when compared to the control strips. This increment in radioactivity represents that portion of C^{14}-dibenamine hydrochloride combined with the alpha adrenergic receptor. It was noteworthy that the control strips contained approximately 75% of the C^{14}-dibenamine hydrochloride present in the experimental strips. The probable reasons for this high content of C^{14}-dibenamine hydrochloride in the control strip became apparent after consideration of the results obtained by labelling procedures 2a and 2b outlined below.

The radioactivity present in the experimental and control strips labelled by procedures 2a and 2b is recorded in Tables 10 and 11. As anticipated a significant increment in radioactivity ($P < 0.01$, procedure 2a; $P < 0.05$, procedure 2b) was noted in the control strips when compared to the experimental strips. This increment in radioactivity represents that portion of C^{14}-dibenamine hydrochloride combined with the alpha adrenergic receptor. The experimental aortic strips labelled according to procedure 2b contained a significantly higher radioactivity ($P < 0.01$)

than the experimental strips labelled according to procedure 2a. The same phenomenon was noted when comparing the control strips labelled by procedures 2a and 2b ($P < 0.01$). This indicated that when the C^{14}-labelled strips remained in contact with the bath fluid for a 3 hour period (procedure 2a) approximately 37% of the C^{14}-dibenamine hydrochloride originally taken up diffuses out of the tissue. This finding provided an explanation for the uptake of some radioactivity by the control strip labelled according to procedure 1. In this procedure the control tissue after treatment with unlabelled dibenamine hydrochloride remained in contact with the bath fluid for 3 hours prior to addition of C^{14}-dibenamine hydrochloride. Hence a considerable portion of the unlabelled dibenamine hydrochloride might have diffused out of the tissue allowing the subsequent uptake of C^{14}-dibenamine hydrochloride

While this work was in progress a report by Dickstein, Silber and Sulman[28,29] of similar studies using dibenamine hydrochloride, randomly labelled with tritium, appeared. In this report it was suggested that cephalin is the tissue acceptor for dibenamine hydrochloride. For this reason the next step in this study should be to examine the distribution of radioactivity in the lipid components of tissues labelled according to the procedures described above. If

cephalin is indeed the alpha adrenergic receptor then it should be possible after labelling aortic strips according to procedure 1 to isolate a cephalin-dibenamine complex from experimental strips containing a higher radioactivity than a corresponding complex from the control strips. The identity of the alpha-adrenergic receptor might also be elucidated by counting the radioactivity of the various components of experimental and control strips labelled according to procedures 2a and 2b. If cephalin is the alpha adrenergic receptor then a cephalin-C^{14}-dibenamine complex should be isolated from control strips but not from experimental strips labelled according to procedure 2a and 2b. It is of interest that Dickstein, Silber and Sulman have not studied the degree of protection of the alpha adrenergic receptor afforded by adrenalin (1×10^{-4}) against dibenamine hydrochloride (1×10^{-5}), the concentration used in their experiments. In view of the report of Furchgott[19] that no protection or doubtful protection of the alpha adrenergic receptors is obtained under these conditions it is likely that the concentrations of adrenaline and dibenamine hydrochloride used were not appropriate.

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